CONCURRENT SESSION 5

Induced Mutations for Enhancing Crop Quality and Nutrition

Induced Mutation-facilitated Genetic Studies of Seed Phosphorus

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Abstract

Both the chemical composition and total amount of seed phosphorus (P) are important to the end-use quality of cereal and legume seed crops, whether for use in human foods or animal feeds. They are also important to the management of P in agricultural production, and to the long-term sustainability of that production. About 75% (±10%) of seed total P is found as phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate). Mutations that block the synthesis or accumulation of phytic acid during seed development, often referred to as *low phytic acid* (*lpa*) mutations, have been isolated in maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and soybean (*Glycine max* L. (Merr.). Chromosomal mapping has identified as many as six non-allelic *lpa* loci in a single species (barley). Studies of *lpa* mutants has enhanced knowledge of the genes and proteins important to phytic acid P metabolism. While there has been substantial research into the biology of P uptake by plants, there has been little progress in the genetics of seed total P. Genetic factors that either decrease or increase seed total P might be of value for both enhancing the end-use quality of seed crops and for optimizing the utilization of P during agricultural production. As proof-of-principle, homozygosity for recessive alleles of barley *lpa*1 both blocks seed phytic acid accumulation by 50% and reduces seed total P by 15%, while having little impact on yield. The current status of *lpa* genetics and current efforts at isolating "seed-total P" mutants, using both forward and reverse genetics approaches, will be described.

Introduction

Engineering the total amount of seed phosphorus (P) or its chemical composition may improve the nutritional quality of grains for use in human foods and animal feeds. Both are also important to the management of P in agricultural production, and to the long-term sustainability of that production [1]. The amount of P annually sequestered in seeds represents a sum equivalent to ~65% of the fertilizer P applied annually, worldwide [2]. The readily available reserves for P fertilizer production worldwide may be consumed within 50 to 150 years [3]. Thus the availability of P for fertilizer may soon prove limiting to world food production. Genetic approaches to engineering optimal seed P amount and chemistry could play an important role both in enhancing food and feed quality and in managing the use and environmental impact of P in agricultural production.

About 75% (±10%) of seed total P is found as phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate or Ins P6, **Fig. 1A**). Seed phytic acid represents a major bottleneck in the flux of P through the agricultural ecosystem. This compound is not efficiently digested and utilized by non-ruminant, monogastric animals such as poultry, swine, fish and humans (nutritional issues related to seed phytic acid are reviewed in [1]). As a result, grains and legumes are a poor source of nutritionally-available P when used in non-ruminant feeds. Excretion of phytic acid P by non-ruminant livestock also represents a significant environmental hazard due to its potential contribution to eutrophication. The seed-derived phytic acid content of foods can function as an anti-nutrient in human diets. Dietary phytic acid binds tightly to nutritionally important minerals such as iron and zinc. Subsequent excretion of these iron and zinc-containing phytate salts can contribute to mineral deficiency, a major public health issue in the developing world. Dietary phytic acid may also have health-beneficial roles, such as an anti-oxidant and anti-cancer agent.

The chemistry of seed total P is not a major issue when grains and legumes are used in ruminant production, such as in dairy or beef production. Ruminants digest most P in feeds, regardless of its composition or chemical form. However, the total amount of seed P is an important issue. As with monogastric production, high levels of P in ruminant waste represents an environmental hazard [4]. This issue has been exacerbated by the increasing use of grain and legume products in biofuels production [5]. An important side product of milling of maize (*Zea mays* L.) for biofuels production is "Distiller's Dry Grains" (DDGs). DDGs usually have a higher total P level than do whole grains, and use of DDGs in livestock production can result in high levels of waste P. One approach to this problem would be to use genetics to reduce the total P of seed, and thus of DDGs. Therefore genetic approaches to reducing seed total P may prove important in reducing the need for P in food and agricultural production. If 65% of the total amount of P removed from crop production fields is removed as seed P, reducing seed P by 25% to 50% has the potential for contributing significantly to enhanced management of this limited resource.

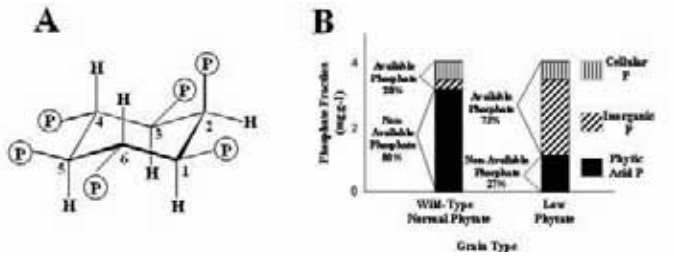

Figure 1 (A) Chemical structure of phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate or InsP6). Numbers refer to the carbon atoms in the myo-inositol (Ins) backbone. "P"=H_2PO_3. **(B)** Seed phosphate fractions in standard "wild-type" grains and legumes and in an example of low-phytate lines or cultivars. "Cellular P" represents the sum of all P-containing compounds other than phytic acid P and inorganic P, such as DNA, RNA, proteins, lipids and carbohydrates. "Available" and "Non-Available" Phosphate refers to nutritional availability for non-ruminants such as poultry, swine and fish, and is based on the assumption that "available P" for non-ruminants approximately equals non-phytic acid P, the sum of all P other than phytic acid P.

Phytic acid genetics

Mutations that block the synthesis or accumulation of phytic acid during seed development, often referred to as *low phytic acid (lpa)* mutations

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(**Table 1**) have been isolated in maize (*Zea mays* L.) [6-10], barley (*Hordeum vulgare* L.) [11-15]; rice (*Oryza sativa* L.), [16-20]; wheat (*Triticum aestivum* L.) [21]; and soybean (*Glycine max* L. (Merr.)) [22-26]. Chromosomal mapping has identified as many as six non-allelic *lpa* loci in a single species (barley) [15].

Studies of the inheritance of the soybean M153 mutant [22] illustrates how new types of genetic screens and new genotypes sometimes illuminate pre-existing genetic variation that was not phenotypically visible. In the case of soybean M153, inheritance studies [23, 24] indicate that the mutant seed phenotype requires homozygosity for recessive alleles at two loci, now termed soybean *pha1* and *pha2* (**Table 1**). However, the generation via chemical mutagenesis of two non-linked recessive alleles and their capture as a "double homozygote" in a single M_2 is a very rare event; it would occur roughly in $1x10^6$ individuals and it is unlikely that this rare individual would then be identified in a screen of only about 1,000 M_2s [22]. It is far more likely that the chemical mutagenesis generated a recessive allele at either *pha1* or *pha2*, and that the soybean population contained a previously existing recessive allele at the second locus that had no clear phenotype of its own.

Progress in identifying the genes perturbed in *lpa* mutants has greatly advanced our knowledge of phytic acid metabolism in plants. The identification of maize *Zmlpa3* and *Zmlpa2* as encoding an Ins kinase and an Ins(1,3,4)P_3 5-/6-kinase, respectively, provided strong evidence for the "lipid-independent" pathway to phytic acid. Previous genetics studies with *Arabidopsis* and other eukaryotes had mostly supported a "lipid-dependent" pathway [27]. Identification of maize *Zmlpa*1 as encoding an "ABC-transporter" represented the first transport function shown to have some role in phytic acid synthesis/accumulation in any organism [9]. Recently, rice *Oslpa*1 was recently shown [18, 20] to encode a protein the closest relative of which is an archaeal 2-phosphoglycerate (2-PGA) kinase, which converts 2-PGA to 2,3-bis-PGA. There are at least two ways the plant homolog of this protein may function in phytic acid synthesis/accumulation. First, 2,3-bis-PGA is a potent inhibitor of phosphatases, the activities of which might block the accumulation of phytic acid. Thus, production of 2,3-bis-PGA might serve to regulate metabolic pathways such that phytic acid accumulates. Alternatively, the protein encoded by rice *lpa*1 might function as an inositol monophosphate kinase, which converts Ins monophosphates to Ins bisphosphates. This type of kinase currently represents the "missing link" in the "lipid-independent" pathway to phytic acid.

Agronomic studies of lpa genotypes

There has been substantial concern over the impact of the low-phytate trait on crop yield. Nearly all *lpa* mutations impact phytic acid synthesis and probably other functions in both seed and vegetative tissues, and thus have negative effects on germination, emergence, stress tolerance, vegetative growth and, ultimately, yield. One of the arguments for the use of genetic engineering in producing the low-phytate trait is that it allows for targeting the trait to specific seed tissues, avoiding impacts on vegetative growth, and thus provides a way for producing high-yielding crops with the low-phytate trait [9]. However, results with two barley *lpa* mutations indicate that mutation genetics might be able to accomplish the same thing. Barley *lpa*1-1 and *lpa*-M640 have been used to breed two released cultivars, "Herald" and "Clearwater," respectively [28, 29]. Both have excellent yields when tested in a variety of environments. Interestingly, both mutations appear to result in a seed-tissue-specific phenotype; they impact phytic acid synthesis only in the aleurone layer, and not in other seed tissues. Perhaps their high yields are due to this seed tissue-targeting which may limit impact on plant/vegetative growth and function.

A second interesting agronomic finding concerns the soybean LR33-Mips and M153 mutants. Both mutants impact field emergence, and other seed function traits like germination, but the effect is much greater if the seed source subsequently tested was produced in tropical environments rather than temperate environments [30, 31]. Seeds of these genotypes produced in temperate environments display much better emergence when planted in either temperate or tropical fields. This effect is not due specifically to the lesion in the LR33 Mips gene, since it is observed in both LR33 and M153, and is not due generally to the low-phytate trait, since a third soybean *lpa* mutation, *lpa*-ZC-2,

Table 1. Low phytic acid loci of cereal and legume crops[a]

Species	Locus	Map Position	Seed Phosphorus (P), Phytic Acid (PA), Inorganic P (Pi), Inositol (Ins) and Ins Phosphate Phenotype
Maize	lpa1	1S	Allele-specific, variable reductions in PA, by 50% to >90%, matched by increased Pi. Increased Ins.
	lpa2	1S	~50%-reduced PA, matched by increased Pi and increased "lower" Ins phosphates such as Ins P4 and Ins P5. Increased Ins.
	lpa3	1S	Largely embryo-specific ~50%-reduced PA matched by increased Pi. Increased Ins.
Wheat	Js-12-LPA	Not Known	~35%-reduced PA, matched by increased Pi. Possibly due to inheritance of alleles at two loci. Alters distribution of P in kernel: increased endosperm P, reduced bran P.
Barley	lpa1	2H	Aleurone/Endosperm-specific ~50%-reduced PA, matched by increased Pi. ~15%-reduced seed total P.
	lpa2	7H	~50%-reduced PA, matched by increased Pi and increased "lower" Ins phosphates such as Ins P4 and Ins P5. Increased Ins.
	lpa3	1H	~70%-reduced PA, matched by increased Pi. Increased Ins.
	lpa-M640	7H	Aleurone/Endosperm-specific ~50%-reduced PA, matched by increased Pi. ~15%-reduced seed total P.
	lpa-M593	4H	~50%-reduced PA. Other linked mutants, all possibly alleles of MIPS gene, have PA reductions from ~10% to >90%.
	lpa-M955	1H	>90%-reduced PA, matched by increased Pi. Increased Ins.
Rice	lpa1	2	~40%-reduced PA, matched by increased Pi.
	Lpa-N15-186	3	~75%-reduced PA, matched by increased Pi.
	lpa-XS110-1	3	~65%-reduced PA, matched by increased Pi.
Soybean	pha1	LG N	~80%-reduced PA, matched by increased Pi, when plants homozygous for both pha1 and pha2. Originally isolated as one of two loci contributing to "M153". Reduced field emergence enhanced if seed produced in tropical environment.
	pha2	LG L	~80%-reduced PA, matched by increased Pi, when plants homozygous for both pha1 and pha2. Originally isolated as one of two loci contributing to "M153". Reduced field emergence enhanced if seed produced in tropical environment.
	lpa-ZC-2	LGB2	~50%-reduced PA, matched by increased Pi and increased "lower" Ins phosphates. Less effect on field emergence than observed for other soybean lpa mutations.
	LR 33-MIPS	Not Known	Ins decreased 60% to 80%. PA decreased ~50%. Reduced field emergence enhanced if seed produced in tropical environment.

[a] Please see the following references for each species: Maize, [6-10]; Barley, [11-15]; Rice, [16-20]; Wheat, [21]; Soybean, [22-26].

does not appear to display this effect [25]. Understanding the underlying biology of this effect would no doubt enhance the understanding of seed biology, and may also be important to the successful development of high-yielding low-phytate crops. This phenomenon hasn't been studied in any of the cereal crop genotypes. Perhaps it might prove important in crops grown in tropical environments, like maize and rice.

Breeding studies with the low-phytate soybean genotypes has also shown that backcrossing and selection for yield within a set of low-phytate lines or segregants can identify lines with improved field emergence and yield [32]. It is not surprising that those mutations that greatly alter metabolism of P, inositol and inositol phosphate, each critical to many processes in cells, tissues and organs of seeds and plants, might impact germination, emergence, and other functions and metabolic pathways that impact yield. For example, a study of genome-wide gene expression during seed development of the barley mutant *lpa*-M955, homozygosity for which nearly abolishes the cell's ability to synthesize phytic acid, found that the expression of a small subset of genes was greatly suppressed during seed development of mutant versus wild-type seeds [33]. These genes are important to carbohydrate metabolism, cell wall metabolism, transport functions and cytokinin and ethylene signalling. These and other lines of evidence suggest that it was premature to expect, and counterproductive to require, that the first crop variants homozygous for *lpa* mutations would perform as well as standard lines, without breeding and selection for productivity and yield. Since these first generation *lpa* lines represent novel genotypes with novel metabolism, it probably should not be surprising that selection for yield might be able to identify favourable non-linked modifiers and allelic variants that restore performance and yield.

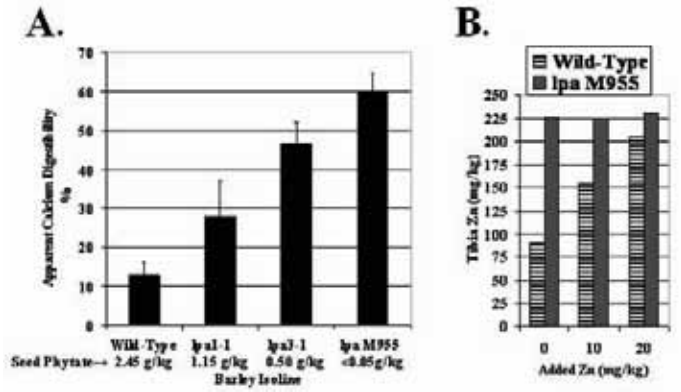

Figure 2 Enhancement of calcium (A) and zinc (B) nutrition in animals consuming diets based on low phytic acid barley lines. **(A)** Trout were raised on four diets consisting of either wild-type barley cultivar that has normal levels of phytic acid, or three barley low phytic acid isolines (lpa1-1, lpa3-1 and lpa-M955), that produce grain with progressively bigger reductions in phytic acid [37]. All diets consisted of 30% barley and contained similar levels of calcium (530 gm kg-1 for lpa1-1 to 590 gm kg^{-1} for W-T). **(B)** Chicks were fed diets consisting of either the wild-type barley cultivar or lpa-M955, whose grain has a reduction in phytic acid of >90%, and that were supplemented with either 0.0, 10 or 20 mg kg^{-1} zinc [38]. Diets contained 60% barely and had similar levels of zinc (~26 mg kg^{-1} zinc).

Human and animal nutrition studies using lpa genotypes

In terms of animal feeds, the primary interest in the low-phytate trait is its high "available P" for non-ruminant animals such as poultry, swine and fish. Many studies (reviewed in [1]) have shown that if formulated properly to take into account lpa seed's high "available" P (illustrated in **Fig. 1B**), non-ruminant animals utilize and absorb a greater fraction of seed total P, and excrete concomitantly less P. In terms of human nutrition, the primary interest in the low-phytate trait has been the potential enhancement in mineral nutrition it may provide for those populations that rely on cereal grains and legumes as staple, bulk foods. A series of studies have shown that fractional absorption of iron, zinc and calcium by human volunteer test subjects is increased 30% to 50% when consuming meals prepared with *lpa* maize versus "normal phytate" maize [34-36]. Two studies with barley *lpa* genotypes and model animal systems (trout and chickens) illustrate some important points concerning the potential enhancement of mineral nutrition made possible by the low-phytate trait. In the first study (**Fig. 2A**) trout were raised on four diets prepared with either "normal phytate" barley (wild-type, 2.45 g phytic acid P kg^{-1}), or with three low-phytate types with increasing reductions in grain phytic acid P (*lpa*1-1, 1.15 mg kg^{-1} phytic acid P; *lpa*3-1, 0.5 g kg^{-1} phytic acid P; *lpa*-M955, <0.05 g kg^{-1} phytic acid P). Barley represented 30% of the total diet. A strikingly linear, inverse relationship between dietary phytic acid and "apparent calcium digestibility", a measure of the animal's retention and use of calcium in the diet, was observed. Calcium retention is critical to bone health and perhaps overlooked in the general discussion of the role of dietary phytic acid in human health and nutrition. Also, it is clear that incremental decreases in dietary phytic acid may result in incremental increases in calcium availability. In other words, there is no critical threshold of reduction in dietary phytic acid necessary to achieve a demonstrated improvement in mineral nutrition.

In the second study (**Fig. 2B**) chicks were fed either wild-type barley or a near-isogenic line homozygous for *lpa*-M955, whose grain has >90% less phytic acid, and these diets were supplemented with either 0.0, 10 or 20 mg kg^{-1} zinc [38]. The bone (tibia) zinc data given in **Fig. 2B** is representative of the results of various measures of zinc nutritional status obtained in the study. The results in Fig. 2B indicate that zinc supplementation was only of value (increased tibia zinc) if the chicks were fed wild-type barley. Chicks fed *lpa*-M955 barley had uniformly high bone zinc and this was not improved by zinc supplementation. Clearly the near absence of phytic acid in the diet allowed for optimal use of the seed-derived dietary zinc. These results also indicate that the level of endogenous zinc in these barleys (~24 mg kg^{-1}) is adequate for optimal growth and health, if dietary phytic acid is greatly reduced, as it is in *lpa*-M955. These sorts of results should be taken into account when public health officials develop strategies for dealing with mineral deficiency in nations with populations that rely on cereals and legumes as staple foods. In this context, while supplementation with iron or zinc may ameliorate either iron or zinc deficiency, reduction in dietary phytic acid may simultaneously enhance both iron and zinc nutrition, and improve nutritional status for calcium and other nutritionally important minerals. This is because dietary phytic acid plays a "global" role in mineral nutrition, simultaneously impacting several nutritionally-important minerals.

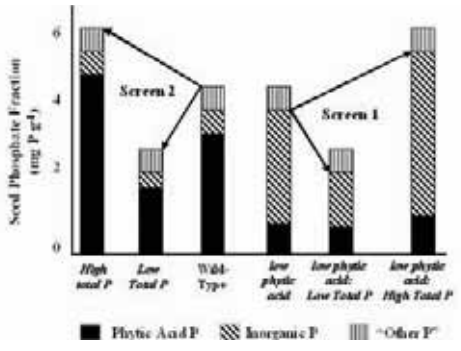

Figure 3 Two types of screens for altered seed total P. Screen No. 1 uses an assay for inorganic P and is for mutations that alter the seed inorganic P phenotype of lpa mutations. Screen No. 2 uses a seed total P assay and will be used with both wild-type and lpa populations. On the right is a parental low phytic acid line and two hypothetical second-site mutations derived from it that either increase or decrease seed total P in a low phytic acid background. On the left is a "normal phytic acid" wild-type parental line and two hypothetical mutations that either increase or decrease seed total P in a "normal phytic acid" background.

Screening for "seed total P" mutations

There has been relatively little progress in the genetics of seed total P. A great deal of research has addressed the biology of P uptake by plants,

and thus the genetics of vegetative or "plant P" level [39]. For example, many mutations have been studied in *Arabidopsis* that impact root or shoot total P. However, there are relatively few reports concerning the genetics of seed total P. Interestingly, homozygosity for recessive alleles of barley *lpa*1 or *lpa*-M640 both blocks seed phytic acid accumulation by 50% and reduces seed total P by 15%, while having little impact on yield [12, 28, 29]. These are the only reports of single-gene mutations that have this effect. We are currently pursuing both forward and reverse genetics approaches to isolating "low-seed-total P" mutants. **Fig. 3** illustrates two types of forward genetic screens. In Screen 1 (**Fig. 3 right**) a high-throughput inorganic P assay is used to screen progeny obtained following the chemical mutagenesis of an *lpa* genotype for any mutation that alters the high-inorganic P phenotype typical of that parental *lpa* genotype. The assumption is that mutations that increase or decrease seed total P will increase or decrease the inorganic P in seed of an *lpa* line, since in an *lpa* genotype inorganic P represents that bulk of seed total P. One interesting thing to note is that the hypothetical "low phytic acid:low total P" mutant illustrated in **Fig. 3** might turn out to have the ideal seed P amount and chemistry for nearly all end-uses since its both low-total P and still "high inorganic P/high available P", as compared with wild-type. The barley *lpa*1 and M640 mutations represent proof-of-principal that such a seed chemistry phenotype is achievable, but we would like to find mutations that reduce seed total P by at least 25%. In Screen 2 (**Fig. 3 left**) following chemical mutagenesis of a normal-phytate, wild-type line, seed is screened directly for mutations that alter seed total P, using a high-throughput assay for total tissue P. Although absolute levels of seed phytic acid are altered in these later hypothetical mutants, they have "normal phytic acid" in the sense that the ability of seeds to synthesize phytic acid is not perturbed, and the proportion of total P found as phytic acid P is not altered.

Conclusions

In addition to the *lpa* forward genetics work described above, much progress has also been made in reverse genetics approaches to issues relating to seed phytic acid. For example, transformation with a bacterial phytase gene targeted to the cytoplasm shows great promise as one approach to developing high-yielding low-phytate crops [40]. Other traditional methods like recurrent selection may also prove valuable in developing low-seed-total P crops [41]. Therefore, many tools obtained from traditional breeding, and forward and reverse genetics will be available to deal with issues having to do with seed total P and its chemistry.

ACKNOWLEDGMENTS

This work was supported by a number of entities including the U.S. Dept. of Agriculture's Ag. Research Service and its CSREES National Research Initiative competitive grants programme, the North American Barley Genome Project, the U.S. National Science Foundation and National Institute of Health, the IAEA, as well as several private companies. Most of the genetic nurseries were grown at locations of the Montana and Idaho USA Agricultural Experiment Stations, and this work would not have been possible without great support from their helpful staffs.

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Biosynthesis and Deposition of Seed Phytate and its Impact on Mineral Bioavailability

S K Rasmussen*, L Bohn, L Josefsen & A M Torp

Abstract

In cereal seeds, phosphorous is primarily deposited in protein storage vacuoles as phytic acid (PA) together with minerals. Even if the same core set of enzymes should exist throughout the plant kingdom, the organization of biosynthesis, translocation, site of accumulation and storage vary among species. PA accumulation in seeds begins shortly after flowering and lasts to seed maturity. During this period the growing plant may be challenged with changes in growth conditions, such as rain, drought, high temperature and pathogens. It has been shown that the individual inositol-phosphate-kinases, Ipk, accept a broad range of substrates and it is also evident that rice and barley Ipks have phosphatase and isomerase activity. These multiple activities provide more degrees of freedom for controlling, and fine tuning, PA biosynthesis during seed development. Isolated phytate globoids from rice and wheat bran have been characterized and K>Mg>Ca>Fe were found as the main minerals. While iron co-purifies with PA in the globoids, this is less evident for zinc, and although copper has high affinity for PA, there is no indication that copper-phytate globoids are the primary storage facility for this element. This difference in seed distribution of iron and zinc must be taken into account in breeding strategies for improving mineral content. The dephosphorylation patterns of pure PA and phytate globoids by wheat phytase have been established, and the kinetics of phytase with either PA or phytate globoids as substrates have been compared. The bioavailability of iron in phytate globoids has been studied using caco-2 cells. PA is an important anti-nutritional factor in the diet of humans because it reduces the bioavailability of iron and zinc. The way food is prepared may solve these problems associated with PA, through the action of endogenous phytases. In addition, low-PA seeds can potentially reduce problems with P management in animal husbandry. Mutational breeding provides a useful way of creating genetic diversity for both PA and mineral bioavailability in cereals.

Introduction

Nutrition, environment and resources

Phytic acid (PA; *myo*-inositol 1,2,3,4,5,6,-hexakisphosphate) is the primary storage compound of phosphorus in plant seeds accounting for up to 80% of the total seed phosphorus and contributing as much as 1.5% to the seed dry weight [1]. Stored PA has an impact on the supply of phosphate and the bioavailability of iron (Fe), zinc (Zn) and other minerals to animals. It has also been implicated in many cellular processes including cancer development. In human nutrition, PA acts as an anti-nutritional factor for, in particular, iron and zinc uptake in the digestive tract, and thus potentially contributes to the 'hidden hunger' of mineral malnutrition. However, PA may also have positive effects on human health, as it has been suggested that PA has anti-carcinogenic effects and the anti-oxidative ability of PA may balance iron ions in solution [2]. **Table 1** shows the wide range of metabolic processes with PA involvement. In animal husbandry, the main problem caused by PA in grain is that phosphate bound in phytic acid cannot be digested by monogastric animals. Thus, phosphate must be supplemented or the enzyme phytase must be added to the feed as a pretreatment. This has an impact on the environment since phosphate and PA-bound phosphorous are released into the fields and can eventually enter the near costal sea areas, lakes and fresh water streams, leading to eutrophication. Finally, a major concern is phosphorus as resource, as it is believed that high-quality rock phosphorous will be exhausted within this century.

Table 1. Involvement of phytic acid in cellular processes

Cancer therapy	Gene regulation	Starch digestibility
Cell differentiation	Efficient export of mRNA	Blood glucose response
Apoptosis	RNA-editing	Prevention of dystropic calcifications
Immune function	Oocyte maturation	Kidney stone formation
Antioxidant	Cell division and differentiation	Lowering cholesterol
Oncogene regulation	DNA repair	Lowering triglycerides
Regulation of Phase I & II enzymes	Cell signalling cascades	Inhibit transcript of HIV-1 genome
Cell proliferation	Ca^{2+} mobilisation and signalling	Prevent plaque formation on teeth
Tumor metastasis	Protein folding and trafficking	
Angiogenesis		
Inflammation		
Controling iron-related oxidative stress, important for men over 20 years	Endo- and exocytosis	

Solutions to the phytic acid in feed

Improving crop plants for feedstuff has obtained most attention, and as natural variation in grain PA content is known to exist, mutational breeding has been an attractive strategy to reduce grain PA content. Many low-phytic acid (*lpa*) mutants have been identified in barley, maize, rice, wheat and soybean [3-8]. These were all identified by an indirect screening procedure based on the assumption that total phosphorus remains constant in the seed, but the distribution between free phosphate and that bound in phytic acid can be modulated. An alternative strategy has been the production of GM crop plants that overproduce a microbial phytase in grain, which can be utilized directly in the feedstuff or added during industrial feed production. However, the most successful solution for the reduction of PA in feed is still the addition of microbial enzymes (phytases) directly to the feed.

Biosynthesis of phytic acid

Significant progress in defining the PA biosynthetic pathways has been made in recent years, and the importance of induced mutations in revealing controlling steps can not be overestimated. Recently, a number of plant genes involved in the pathway have been cloned and characterized. **Table 2** summarizes those originating from the cereals, maize, rice

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **162-164**

and barley. The *myo*-inositol monophosphate synthase gene has been identified in several plant species [9-11]. The encoded protein converts glucose-6-phosphate to inositol-3-phosphate. This appears to be the first committed step in a sequential phosphorylation of *myo*-inositol to PA and provides the only source of the inositol ring. An inositol kinase that produces inositol monophosphates from inositol, and which is required for normal PA accumulation, has been cloned in maize (*Zea mays*) [12]. An inositol polyphosphate kinase has been characterized in maize [13] and *Arabidopsis thaliana* [14], and most recently, two genes involved in late phosphorylation steps to PA, have been cloned and characterized in *Arabidopsis* [15-18]. A detailed characterization of a multifunctional inositol phosphate kinase from rice and barley belonging to the ATP-grasp superfamily show the highest activity towards Ins (3,4,5,6)P_4, which is likely the primary substrate [19]. Based on the cloned genes, combined with the knowledge from mammalian and yeast systems, several pathways to PA have been suggested to exist in plants, however, these still need to be experimentally confirmed.

In addition to kinases and synthases, unexpected genes have been found to be involved in the accumulation of PA during seed development. This includes an ABC-transporter [20] from maize and rice and 2-phosphoglycerate kinase from rice [21, 22]. This shows that random mutations may help us to uncover as yet unknown controlling steps in PA metabolism and sequestration.

Enzyme	Species	Gene	Accession No[1]	References
Myo-inositol-1 phosphate synthase (MIPS)	Rice	*RIN01*[2]	AB012107 LOC_Os03g09250	[10, 29]
	Rice	*RINO2*	AK069323 LOC_Os10g22450	[31]
	Barley	*MIPS*	AF056325	[9]
	Maize	*MIPS*[3]	AF056326	[9]
Inositol monophosphatase (IMP)	Rice	*OsIMP-1*	AK071149 LOC_Os03g39000	[31]
	Rice	*OsIMP-2*	AK103039 LOC_Os02g07350	[31]
	Barley	*HvIMP-1*	AY460570	[33]
Myo-inositol kinase (MIK)	Maize	*ZmLpa3*[2,4]	AY772410	[12]
	Rice	*OsMIK*[2]	AC118133 LOC_Os03g52760	[12, 28]
Inositol 1,4,5-tris-phosphate kinase (IPK2)	Rice	*OsIPK2*[5]	AK072296 LOC_Os02g32370	[31]
Inositol 1,3,4,5,6-penta*kis*-phosphate 2-kinase (IPK1)	Rice	*OsIPK1*[5]	AK102842 LOC_Os04g56580	[31]
	Maize	*ZmIPK1A*[4]	EF447274	[30]
	Maize	*ZmIPK1B*	EF527875	[30]
Inositol 1,3,4- tri*kis*phosphate 5/6-kinase (ITP5/6K)	Maize	*ZmIPK*[2,4]	AY172635	[13]
	Rice	*OsIPK*[4,6]	NP922861 LOC_Os10g42550	[19]
	Barley	*HvIPK*[4]	AM404177	[19]
	Rice	*OsITP5/6K-1*	AK106544 LOC_Os10g01480	[31]
	Rice	*OsITP5/6K-2*	AK100971 LOC_Os03g12840	[31]
	Rice	*OsITP5/6K-3*	AK067068 LOC_Os03g51610	[31]
	Rice	*OsITP5/6K-4*	AK071209 LOC_Os02g26720	[31]
	Rice	*OsITP5/6K-5*	AK059148 L0C_Os10g42550	[31]
	Rice	*OsITP5/6K-6*	AK102571 LOC_Os09g34300	[31]
ABC-transporter (MRP)	Maize	*ZmMRP4*[2]	EF586878	[20]
	Rice	*OsMRP13*	Os03g04920	[20]
2-Phosphoglycerate kinase (2PGK)	Rice	*Os2PGK*[2]	LOC_Os02g57400	[21, 22]
	Rice	*Os2PGK*	LOC_Os09g39870	[21]

Table 2. Cloned genes involved in the biosynthesis and accumulation of phytic acid in cereals

(1) Genebank accession number (nucleotide) and TIGR locus identifier (for rice genes); **(2)** Mutants or transgenic antisense plants for the gene show lpa phenotype; **(3)** Map location of cloned gene corresponds with that of a lpa mutant; **(4)** Recombinant protein show kinase activity; **(5)** Positive complementation test in yeast lacking the corresponding gene; **(6)** *OsIPK* [19] and *OsITP5/6K-5* [31] are likely to be the same gene.

Phytic acid globoids and mineral content

Phytic acid accumulates during seed development in electron-dense particles named globoids, which also store minerals. These globoids were purified from wheat bran and the content of minerals quantified by inductively coupled plasma-mass spectrometry [23]. The main elements, in concentration order, were K>Mg>Ca>Fe and although copper has high affinity for PA there is no indication that globoids are the main store for this element. Interestingly, whereas iron co-purifies with phytic acid globoids, this was not found for zinc, which is in agreement with the notion that zinc is stored in other compartments of the developing seed.

Degradation of phytic acid

The stored phosphorus in PA is believed to be remobilized during germination to provide phosphorus to the emerging seedling. Some plant species store phytases in the resting grain, ready to initiate dephosphorylation of phytic acid. Phytases have been purified and characterized from seven cereals and 16 other plant species [2]. Phytases are specialized phosphatases [24,25] that utilize phytic acid as substrate. In a detailed biochemical study, wheat bran phytase was shown to initiate at the C6 and C3 positions of the inositol ring [23]. It is noteworthy that the subsequent dephosphorylation of inositol-phosphate is ordered and sequential. It is not random.

Phytic acid and bioavailability of iron

CaCo-2 cells provide an ideal way of elucidating a simple picture of the interactions between phytase and its substrate to investigate iron bioavailability in globoids. The natural PA: iron ratio of wheat globoids is 51:1, and a ratio lower than 27:1 is required for significant iron bioavailability (Bohn, unpublished results). Results from other investigations of this phenomenon have revealed a threshold of 5:1 for PA: Fe^{2+} complexes [26] and 10:1 using pure PA: Fe^{3+} complexes [27]. The bioavailability of Fe at a ratio of 12:1 from globoids suggests that factors that increase iron absorption are present in the solution. Pepsin degradation of the proteins may reveal peptides as weak chelators to maintain iron solubility. Furthermore, as PA has stronger binding to other minerals, such as copper, zinc and manganese, these cations could be binding competitively to the PA, thereby leaving iron free for absorption. Some of these cations do, however, also inhibit iron uptake by the intestines, and the impact of their binding to either PA or the DMT1-transporter remains unknown. Taking these results further would involve the investigating of the bioavailability of other minerals from globoids, such as zinc or calcium, in CaCo-2 cells whilst altering PA concentrations. Once the bioavailability of the elements in globoids are understood, similar experiments could be performed using bran or whole-wheat bread, to explore the inhibition of mineral uptake by fibers, cell walls, starch etc. This would provide information on the optimal treatment of cereals to improve the uptake of minerals from them without depleting PA completely.

ACKNOWLEDGEMENTS

This project was supported by the Danish Research Council for Technology and Production Sciences, The Danish Council for Strategic Research and HarvestPlus.

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Revealing the Complex System of Starch Biosynthesis in Higher Plants Using Rice Mutants and Transformants

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Abstract

Starch is the end product of photosynthesis and a primary material for food and industrial uses. Starch has a variety of distinct physico-chemical properties such as gelatinization and pasting properties, and these features are strongly related to the molecular structure of amylopectin and the formation of starch granules, whose morphology depends on the plant species. The multi-dimensional, unique structure of starch is achieved by concerted reactions catalyzed by multiple isozymes of a set of enzymes that include starch synthase, starch branching enzyme and starch debranching enzyme. The action mechanism of each of these isozymes is currently being studied. This paper summarizes recent results of biochemical and genetic analyses of starch biosynthesis in rice endosperm obtained from various mutants and transformants, and discusses ideas about the regulation of starch biosynthesis in plants.

Starch is glucose polymer with two α-glucosidic linkages, linearly linked α-1,4-glucosidic chains are branched by α-1,4-glucosidic linkages, and it comprises linear or rarely branched amylose and highly branched amylopectin. Amylopectin has a distinct highly ordered structure called a "tandem-cluster structure," in which most of side chains are arranged in parallel and neighboring chains form double helices when linear portions of facing chains reach the length equivalent to degree of polymerization (DP) ≥ 10. The formation of double helices in the amylopectin cluster dramatically induces its hydrophobicity and crystallinity. These specific features of amylopectin fine structure are enabled by the localization of branch positions within the restricted region of the cluster. The starch synthesis system has developed during the evolution of plants and key enzymes involved in the construction of amylopectin tandem-cluster structure have differentiated into multiple isozymes with distinct functions, whereas in glycogen synthesizing organisms, such as bacteria and animals, no such functional differentiation in glycogen synthesis enzymes has occurred (**Fig. 1,** [1-5]).

Starch branching enzyme (BE) plays a very important part in the formation of branches in amylopectin molecules. Green plants are known to have two types of BE isozymes, BEI and BEII. In addition, BEII is further differentiated into BEIIa and BEIIb isoforms in cereals although BEIIb is usually specifically expressed in endosperm while BEIIa is ubiquitously present in every tissue. Our biochemical studies of three mutants of rice that are defective in BEI, BEIIa, and BEIIb, respectively, strongly suggest that the role of BEIIb is highly specific in synthesizing branches located on the basal portion of the crystal zone (referred to as the crystal lamellae) of the cluster because BEI and BEIIa can hardly complement its role in its absence (**Fig. 2, [1, 6, 7]**). On the other hand, BEI plays an important role in forming branches that are positioned at the basal part of the cluster in the less crystalline zone (referred to as the amorphous lamellae), and those which link the clusters, but its role can be largely complemented by BEIIb and BEIIa in the BEI mutant [1, 8].

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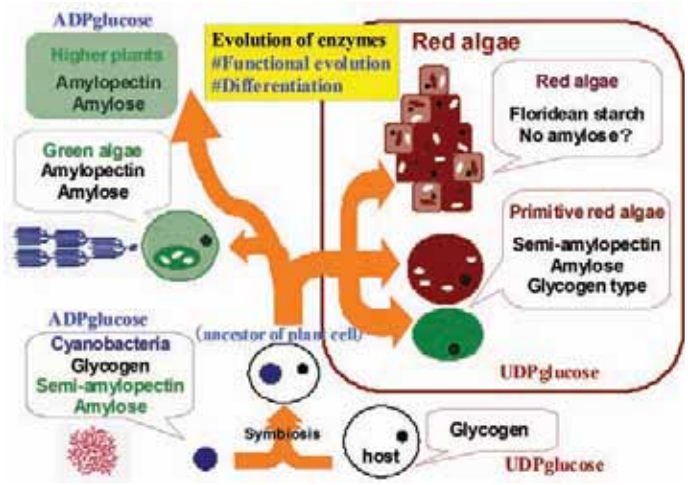

Figure 1 Schematic representation of the structure and synthesis of α-glucans during plant evolution. It is noted that ADP glucose is used in cyanobacteria, green algae and higher plants as precursor of glucans, while UDP glucose is used in red algae. During the evolution of starch biosynthesis, enzymes are thought to have evolved functionally and the number of isozymes to have increased.

Although the activity of BEIIa accounts for about 20% of the total BE activity in rice endosperm [9], the specificity of its function is likely to be poor because no significant changes in the structure of amylopectin and the physicochemical properties of starch granules are found in the BEIIa mutant [1, Nishi, *et al.*, unpublished data]. This data indicates that the branches within the cluster can be divided into at least two groups which are distinguished by different BE isozymes.

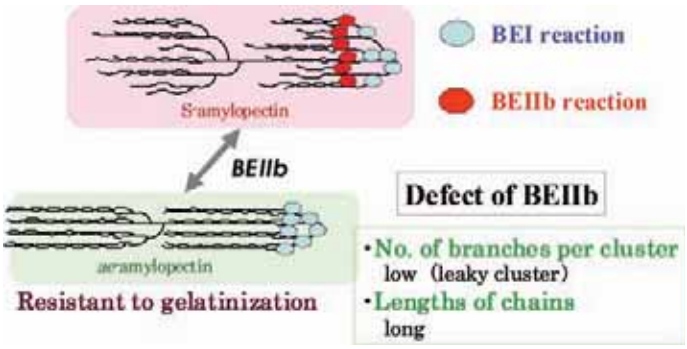

Figure 2 The schematic representation of distinct functions of BEI and BEIIb isoforms present in rice endosperm. It is noted that this scheme can explain why ae-amylopectin can be formed in the absence of BEIIb.

Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009,**165-167**

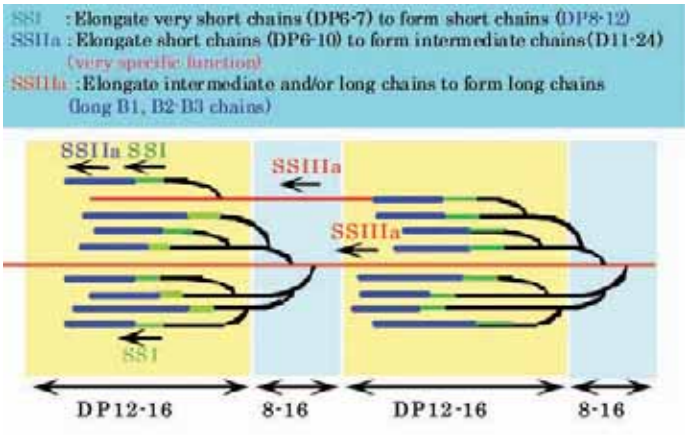

Figure 3 The schematic representation of distinct functions of three SS isozymes in rice endosperm. The figure shows that the synthesis of amylopectin cluster can be performed by concerted reactions of SSI, SSIIa and SSIIIa.

Rice has three major starch synthase (SS) isozymes, SSI [1, 10], SSIIa [1, 11] and SSIIIa [1, 12] in developing endosperm. We recently determined the distinct properties of each isozyme; SSI, SSIIa, and SSIIIa are responsible for the synthesis of very short chains with DP~8-12, intermediate chains with DP≤~24, and long chains with DP≥~20, respectively (**Fig. 3**). Since each SS isozyme is thought to recognize the chain-length from the non-reducing end to the branch point of the α-1, 4-chains and elongates it until its range is exceeded, the maximal length to which each SS isozyme can elongate the chain is strictly restricted and differs between isozymes. This feature might be important for each cluster to obtain a fixed length of DP27-28.

Starch debranching enzyme (DBE) plays an essential role in the synthesis of the amylopectin cluster by trimming the shape of the cluster, because in its absence amylopectin is replaced by phytoglycogen [1, 13]. Plants have two DBE-types, isoamylase (ISA) and pullulanase (PUL), and generally three ISA isozymes (ISA1, ISA2 and ISA3) and one PUL isoform. ISA activity involved in starch biosynthesis is due to the ISA1-ISA2 hetero-oligomer in potato tuber and Arabidopsis leaves [14], while the endosperm of rice and maize contains both the ISA1 homo-oligomer and the hetero-oligomer [15]. Although the details of how DBEs are involved in the synthesis of amylopectin are unknown, it is thought that these enzymes remove improper branches that interfere with the formation of double helices [1] and accelerate the crystallization of the cluster [16].

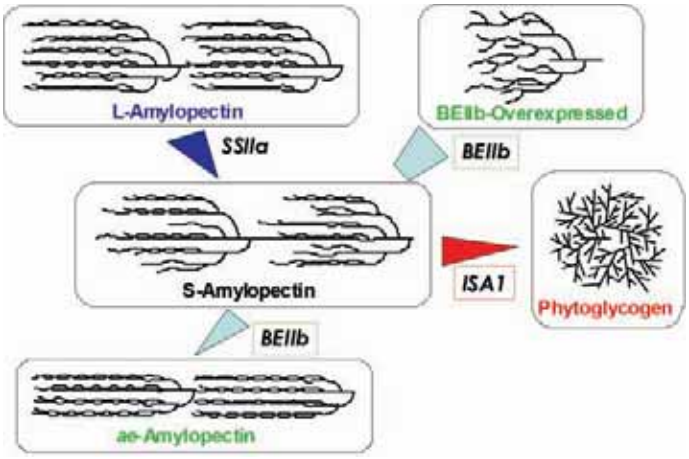

Figure 4 Schematic representation of changes in the amylopectin cluster structure induced by different levels of key enzyme isoforms (cluster-world). Note that the type of the cluster structure is determined by the enzyme activity of the individual enzyme isoform.

Detailed analyses of changes in the structure of amylopectin and the physicochemical properties of starch granules in rice endosperm caused by the absence of each isoform of BE, SS and DBE, have established that individual mutants exhibit distinct characteristics in terms of the starch structure and properties depending on the enzyme activities inhibited. These patterns reflect the specific functions of each enzyme in starch biosynthesis in rice endosperm and enable us to predict how, and to what extent the structure and properties of starch can be engineered by manipulating the genes encoding starch-synthesizing enzymes (**Fig. 4**). Our results suggest that numerous rice mutant lines could be used for industrial purposes in the future by producing novel starches in the endosperm.

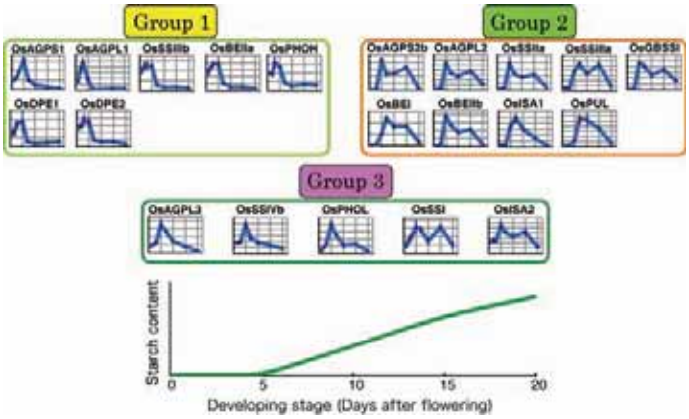

Figure 5 The pattern of changes in transcript levels of starch synthesizing genes during endosperm development of rice. Group 3 genes are expressed from the early stages to the latter stage.

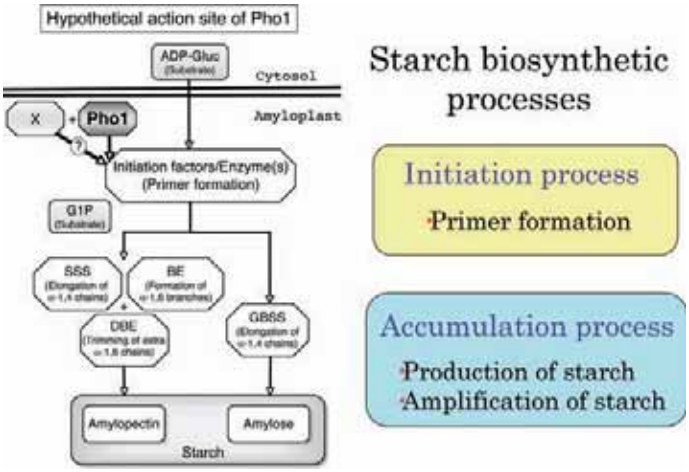

Figure 6 The schematic representation of the two distinct processes of starch biosynthesis and the possible role of Pho1 in rice endosperm. We assume that Pho1 plays a crucial role in the initiation process by synthesizing primers of amylopectin molecules and/or starch granules.

Transcriptome analysis has established that changes in transcript levels of genes encoding starch-synthesizing enzymes in rice endosperm can be divided into three temporal patterns [17]. One group of genes (Group 1 in **Fig. 5**) are highly expressed in the very early stages of endosperm development (days after pollination, DAP, up to about five days) prior to the onset of rapid starch production, whereas the other group genes (Group 2 in **Fig. 5**) are expressed more when starch accumulation in the endosperm is at its greatest (**Fig. 5**).

Recent studies using rice mutants lacking plastidic glucan phosphorylase (Pho1) showed that the loss of Pho1 resulted in a severe reduction

in starch accumulation in some seeds, while other seeds have plumped shape, suggesting that Pho1 plays a crucial role in the initial stage of starch biosynthesis such as initiation of glucan molecules and starch granules [18].

These results strongly suggest that starch biosynthesis is composed of two distinct processes, namely the initiation process of starch biosynthesis, in which glucan primers are synthesized and/or the initial core of starch granules are formed, and the accumulation and/or amplification process, in which the number of starch molecules and starch granule are amplified, and that these processes are regulated by different mechanisms and include different sets of enzyme isozymes (**Fig. 6**).

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Mutants Pave the Way to Wheat and Barley for Celiac Patients and Dietary Health

D von Wettstein

Abstract

Wheat has two major nutritional problems for the consumer: (1) The flour or pasta produced from the grain is not acceptable to congenital celiac patients and may induce intolerance of dietary "gluten" in people later in life. (2) The grain is highly deficient in the essential amino acid lysine. Currently there is only one treatment for sufferers of celiac disease: the complete exclusion of wheat, barley and rye grains from their diets. Celiac disease is caused by an autoimmune reaction against undigested proline/glutamine rich peptides (epitopes) that are taken up through the intestinal mucosa and initiate an autoimmune response in human leucocyte antigen DQ2- or DQ8-positive individuals. This leads to chronic erasure of the microvilli of the intestinal epithelium and to permanent intolerance of dietary "gluten." Cereal prolamins are of two types: high molecular weight glutenins (HMWG) with a molecular structure of elastic fibrils that form dityrosine cross-links during dough formation and baking, and gliadins. The gene promoters of the gliadin-type proteins are silenced by DNA methylation in vegetative tissues. This methylation is removed during grain development to permit protein synthesis. Inhibition of the demethylation by mutation specifically inhibits the synthesis of the gliadin-type proteins and only proteins consisting of elastic fibrils are produced. As a proof of principle, a barley cultivar called Lysiba already exists that has such a mutation and provides the rationale for creating wheat varieties by mutation of the 5-methylcytosine deglycosylases in the endosperm. Celiac patients are sensitive to a wide variety of different epitopes, which are located in the gliadin-type prolamins. Gliadin-type prolamins are of no importance for baking because wheat HMW glutenin has been shown to be alone sufficient to produce high quality breads.

Introduction

Celiac disease is the most common food-sensitive enteropathological condition in humans and it is caused by an autoimmune reaction against certain wheat, barley and rye grain storage proteins. In human leukocyte antigen (HLA) DQ2- (or DQ8-) positive individuals' exposure to these "gluten" proteins can lead to a painful chronic erasure of the microvilli of the epithelium in the intestine and to a permanent intolerance of dietary prolamins. The autoimmune response results from the resistance to digestion of certain proline/glutamine-rich peptides (epitopes) in the prolamins by gastric, pancreatic and brushborder membrane proteases. Peptides like PFPQP**Q**LPY are taken up through the intestinal mucosa into the *lamina propria* and initiate the autoimmune response [1]. Celiac disease is commonly detected in congenital cases with severe symptoms in early childhood. In an increasing number of patients, symptoms arise only later in life as a result of bread and pasta consumption. If untreated, celiac disease may cause increased morbidity and mortality. Despite its prevalence in most populations comprising 24.4 million registered celiac individuals world-wide [2], the only effective therapy is strict dietary abstinence from these food grains [3]. However, because of the multiple presentations of the disease, many sufferers of this disease have not been formally diagnosed with it and estimates suggest that for every registered celiac there are 50 unrecognized individuals.

Our aim is to eliminate the prolamins from wheat grain that contain the majority of epitopes causing celiac disease. Eliminating these proteins will also address the other major quality problem for the consumer of wheat products: the imbalance in the amino acid profile of wheat proteins. Wheat grain is especially low in lysine which is the most limiting amino acid in cereal proteins for humans and monogastric animals. Because the prolamin protein families we are targeting are very lysine poor, their elimination will lead to a considerable increase in grain lysine content with concomitant improved nutritional quality, which will be beneficial for all consumers of wheat products. The highly homologous storage proteins of wheat, barley and rye called prolamins fall into two groups: one group, represented by the lysine poor gliadins and low molecular weight (LMW) glutenins of wheat, contain the overwhelming majority of the protein domains (epitopes) causing the celiac response and are dispensable for baking, and can therefore, be removed. The other group represented by the wheat high molecular weight (HMW) glutenins are alone required for dough formation and baking, and therefore must be retained. The molecular structure of these two types of prolamins is very different and their genes are turned on and off by two fundamentally different mechanisms, which provides the strategy for elimination of the gliadins and LMW glutenins but preservation of the HMW glutenins. The genes for gliadins and LMW glutenins are silenced by DNA methylation of their promoters in vegetative tissues. The promoters have to be de-methylated at the beginning of endosperm development to permit their transcription and the synthesis of the encoded prolamins. The aim is to silence these genes permanently in the endosperm by mutations in the enzymes carrying out the demethylation. A fully viable mutant with these characteristics has been obtained in barley. The six wheat genes encoding the elastic fibrils of the HMW glutenins that form dityrosine cross-links during dough formation and baking and pasta production are protected against DNA methylation in all tissues by a CpG island in their promoter. They are turned on by synthesis of transcription factors or removal of repressor proteins at the beginning of grain filling.

Preliminary studies

Only HMW glutenin is required for baking

Ingo Bauer [4] has transformed yeast with the wheat HMW glutenin genes (*HMWDx5* and *HMWDy10*) and synthesized in fermenters gram quantities of the HMW glutenins. The HMW glutenin proteins were extracted and highly purified. From the dough made with commercial wheat flour, all gliadins, LMW and HMW glutenins were removed by washing. The residues containing starch, soluble protein, fat, fibers and minerals were ground together with the purified HMW glutenin protein into flour, kneaded to dough and baked. The dough showed excellent elasticity and resultant bread rolls had the desired volume and internal structure.

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **168-171**

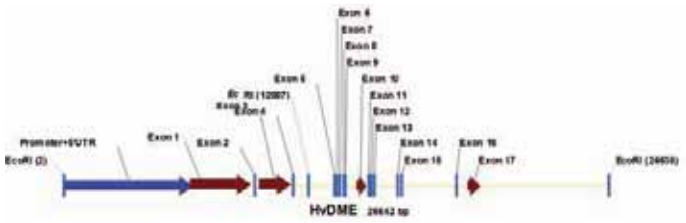
The lys 3a regulatory mutant in barley points the way to celiac safe wheat

The recessive high lysine mutant 1508 (*lys 3a*) was selected after mutagenesis of the cultivar Bomi with ethyleneimine. Its lysine content in the grain protein was increased by 44% [5]. Feeding trials with rats and pigs revealed superior nutritional quality. Breeding by crossing the mutant gene into other genotypes produced the cultivars Piggy, Lysimax and Lysiba with high lysine and improved yield and thousand grain weights [6, 7]. The increased lysine content is primarily due to a reduction in prolamins and an increase in free amino acids. Ultrastructural information on the development of protein bodies in the barley endosperm of wild type and the high lysine mutants *lys* 3a and *hor* 2ca (Riso 56) was provided by electron microscopy [8]. The hordein polypeptides are synthesized on the polysomes of the endoplasmic reticulum, co-translationally transferred into the lumen of the endoplasmic reticulum and then into the storage vacuoles, where they are compacted into the protein bodies. Mutant *lys*3a produces very little B- and C- hordeins. Protein bodies of *lys*3a contain only D-hordein characterized by the resilin/elastin like fibrillar structure. The proposed pathway of protein body formation has been verified using isolated endoplasmatic reticulum, isolated protein bodies and by localization studies with monoclonal antibodies for individual hordein polypeptides [9]. Cloning and sequencing of structural genes for B-, C- and γ-hordein polypeptides have corroborated that the *Hor 1*, *Hor* 2, and *Hor* F loci each consist of a family of closely linked structural genes for these proteins, with each gene having its own promoter [10]. Some of these genes contain stop codons, but transient transformation experiments revealed that expression of such a gene in the endosperm can take place by amber codon suppression [11]. The deposition of the prolamins in the protein bodies of wheat is similar to that in barley [12].

The nature of the high lysine mutant *lys* 3a was clarified [13 for review]. Transcripts encoding B-hordein, C-hordein and protein Z are practically absent in the developing endosperm of the mutant, while the transcript levels for D-hordein, glyceraldehyde-3-phosphate dehydrogenase and histone are normal. Mikael Blom Sørensen [14] showed by genomic sequencing and ligation mediated PCR that cytosines of 10 CpGs in the promoters of the B-hordein genes and 4 CpGs of the adjacent coding region of the gene are hypomethylated in the endosperm but fully methylated in the leaf. In the developing endosperm of the mutant demethylation of the B-hordein promoter does not take place. Genomic sequencing of the D-hordein gene promoter using bisulfite treated DNA revealed a CpG island and confirmed that the promoter is unmethylated in the leaf as well as in the endosperm both in the wild type and in the mutant [15].

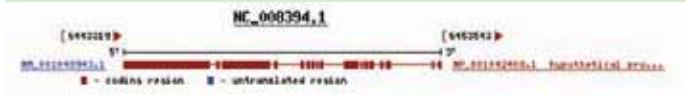
This form of transcriptional control by DNA methylation and demethylation was rediscovered in 2002 in *Arabidopsis* in connection with studies of genetic imprinting. The 5-methylcytosine deglycosylase, named *DEMETER*, was cloned and the sequence identified for *Arabidopsis* [16, 17] and rice (Gene Bank Accession number AF521596). The *Arabidopsis* enzyme (1,729 aa) is a bifunctional helix-hairpin-helix DNA glycosylase with a proline-rich loop containing aspartic acid (D1304) and lysine (K1286) serving as catalytic residues in the 5-methylcytosine excision reaction [18]. An apyrimidinic lyase activity nicks the DNA generating a 3′-hydroxyl to which a DNA repair polymerase adds an unmethylated cytosine. A ligase completes the repair process by sealing the nick. In the barley endosperm this demethylation process and initiation of transcription takes place immediately prior to the accumulation of the hordein B, C and γ transcripts [19].

Thus, two categories of promoters for endosperm-specific gene expression can be distinguished; one that is silenced by methylation in vegetative tissues and has to be demethylated before activation of transcription can take place; the other solely dependent on removal of repressors or induction of transcription factors specific for the endosperm. In hexaploid wheat, in each genome, on the long arm of chromosome 1, there are two linked genes encoding HMW glutenin. The promoter of the wheat HMW glutenin gene *Glu-1D-1b* [20], Acc.X12928) is 89% identical in nucleotide sequence to the barley D-hordein promoter and contains a similar number of CpG dinucleotides [15, 21]. It is therefore expected to be unmethylated in the developing endosperm and the other organs of the wheat plant. This provides the rationale for investigating, if in wheat –by analogy to barley– the gliadins and LMW glutenins can be eliminated by transcriptional silencing without affecting the synthesis of the HMW glutenins.



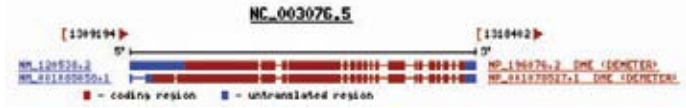
HvDME

mRNA	bp	Exons	Protein	aa	Exons
	5943	17		1981	17



OsDME Os01g0217900 hypothetical protein [*Oryza sativa* Japonica Group]

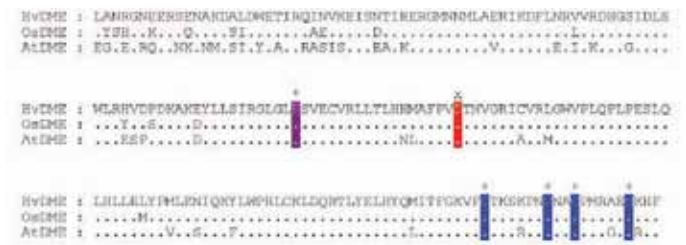
mRNA	bp	Exons	Protein	aa	Exons
NM_001048943.1	5784	17	NP_001042408.1	1927	17



AtDME DME (DEMETER) [*Arabidopsis thaliana*]

mRNA	bp	Exons	Protein	aa	Exons
Splice variant 1 NM_120538.2	6963	19	NP_196076.2	1729	18
Splice variant 2 NM_001085058.1	6475	20	NP_001078527.1	1987	19

Comparison of conserved Endo3/FES domain



An asterisk marks the lysine residue that is diagnostic of a glycosylase/lyase activity, x indicates the conserved aspartic acid residue in the active site, and + the cysteine residues for binding [4Fe–4S] cluster. DME: DEMETER; Hv: *Hordeum vulgare*; Os: *Oryza sativa*; At: *Arabidopsis thaliana*.

Figure 1 The structure of the barley DEMETER gene (HvDME) compared to the rice gene (OsDME) and two gene splice variants of the *Arabidopsis* gene (AtDME). Below are the amino acid sequences of the conserved endonuclease / FES domain of the three enzymes.

Is the lys 3a mutation in the barley Demeter gene?

We have used the Plant Transcript Assembly Report TA38047_4513 from the TIGR Database covering 2312 nucleotides corresponding to *DEMETER* of *Arabidopsis thaliana* with 77.47% nucleotide identity and 56.52% coverage to design a forward and reverse primer using primer3 software. Primers TA38047for 5'-TGTGCGTCTTTTGACACTCC-3' and TA38047rev 5'-GCTCGTACAATGTCCGTTGA-3' were used to amplify barley genomic DNA or cDNA. PCR products were visualized by gel electrophoresis. The primers yielded in a PCR reaction on barley genomic and cDNA respectively a 342 and 187 nucleotide fragment. The sequence of the genomic fragment covers introns no. 6 and 7 and adjacent domains with a nucleotide coding identity between rice and barley of 91.6%. The fragment was radioactively labelled and hybridized to the filters of the cv. Morex six-row barley BAC library. The probe hybridized to a single BAC clone, which has been subcloned and sequenced as summarized in **Fig. 1** (R. Brueggeman, G. Langen, J.Pang, unpubl.). With its guidance we are sequencing the *DEMETER* genes of the *lys3a* mutant and its mother variety Bomi. A suspected mutation will be verified by expressing wild type and mutant *DEMETER* cDNA clones in *E.coli* with a His-tag. The resulting proteins will be purified on a Ni^{2+}-NTA column and their activity tested with methylcytosine containing double stranded oligonucleotides [18]. A mutation affecting the activity of the *DEMETER* protein will be recognized by its inability or reduced ability to excise 5-methylcytosine.

Analysis of promoter methylation/demethylation of genes encoding wheat prolamins

An analysis of the presence or absence of CpG islands revealed that the HMW-glutenin genes Glu-A1-2, Glu-B1i, Glu-1D-1b, Glu-1D-2b, Glu-1Ax1 and Glu-1Dy10 contained a CpG island in their promoter (S.Rustgi, unpublished). Thus the DNA of all six HMW-glutenin genes in the three genomes of bread wheat are expected to be unmethylated in the vegetative organs as well as in the developing endosperm and their transcriptional activity will be retained upon specific silencing of the wheat *DEMETER* genes in the developing endosperm. A CpG island was on the other hand not detected in the promoters of three α-gliadin genes (Gene Bank Acc. numbers X01130, X02538, X02540) and the LMW-glutenin gene GluD3-3 (DQ357058) and we expect therefore that the transcriptional activity of the gliadin and LMW glutenin genes can be eliminated in the endosperm by silencing the wheat *DEMETER* genes.

Three genes for wheat 5-methylcytosine DNA deglycosylases

Based on synteny studies with rice, two homologues of the *DEMETER* gene were located on wheat chromosomes 3DS (bin 3 FL 0.24-0.55) and 3AS (bin 4 FL 0.45-1.00), respectively. It will have to be determined if there is also a *DEMETER* gene in the B genome. Partial sequences of the Demeter domains in wheat, rice and barley databases have been aligned and display significant homology (S.Rustgi, unpublished). For wheat we have a 3′ sequence of 981 nucleotides (pos. 6033-7014) from a 5-methylcytosine deglycosylase. With a radioactive labelled probe for this sequence we screened the hexaploid wheat (*Triticum aestivum*) BAC library containing 1.3 million clones, representing 7x coverage of the genome [22] and identified 12 BAC clones containing the targeted gene(s) (R. Brueggemann, C.G. Kannagara, unpublished). They are being fingerprinted by digestion with the *Hind*III restriction enzyme to identify the unique clones. It is expected that we can sequence and assemble the three expected genes for the 5-methylcytosine deglycosylases from these BAC clones.

Transient silencing of genes in the developing wheat spike using the barley stripe mosaic virus (BSMV)

It is likely that the three wheat *DEMETER* genes will contain identical DNA sequences in their coding regions and that these will be suitable for transcriptional silencing of all three genes, thereby preventing the synthesis of the gliadins and LMW glutenins encoded in the three genomes in the developing endosperm. The *barley stripe mosaic virus* (BSMV) gene silencing procedure with constructs containing sense and antisense DNA fragments for phytoene desaturase [23] has been adapted to inhibit endosperm granule bound starch synthase (encoded by waxy gene) in the developing caryopses of the wheat spike (H. Pennypaul and K.S. Gill, unpublished), and thus provides a model for studying silencing of the DEMETER genes. BMSV is a positive-sense RNA virus with a tripartite genome consisting of RNAs α, β and γ. The three infectious RNAs are prepared from cDNA clones by *in vitro* transcription using T7 DNA-dependent RNA polymerase [24]. The modifications of BSMV for silencing or overexpression are as follows: The gene for the virus coat protein in the β-genome has been deleted. The γ-RNA cDNA clone was modified to allow insertion of sense or antisense fragments after the stop codon of its γb gene into a *Pac*I/*Not*I cloning site. As a test, 200 bp fragments of granule bound starch synthase *GBSS1* and *GBSS2* (*Waxy*) were used to inhibit the synthesis of amylose in the developing endosperm. The infectious RNAs were rubbed on to the flag leaf prior to heading of the spike and arrived at eight days after fertilization during the development of the endosperm. They reduced the synthesis of amylose by 50%. It is intended to insert into the cloning site of the γ genome cDNA fragments of the wheat DNA 5-methylcytosine deglycosylase in sense or antisense direction and similarly infect the flag leaf. This will allow the recombinant viral RNA to arrive in the endosperm at 8 DAP, i.e., at the beginning of the gliadin synthesis and promoter demethylation. We will evaluate the inhibition of the synthesis of the wheat gliatin genes by PAGE analysis of endosperm proteins and transcripts by real time RT PCR and expect to find inhibition of gliadin and LMW glutenin synthesis but not for accumulation of HMW glutenins.

Wheat plants with mutations in the three 5-methylcytosine DNA deglycosylase genes

The three 5-methylcytosine deglycosylase genes of the three wheat genomes will have in their introns and/or exons sequence differences that distinguish them sufficiently to devise homoeolog-specific primers for TILLING (Targeting Induced Local Lesions in Genomes) of these genes in hexaploid Express spring wheat and Kronos tetraploid pasta wheat [25, 26]. In cooperation with Charles P. Moehs, Arcadia Biosciences Inc. the DNA of M_2 individuals of these two varieties will be screened with the specific primers identified for the three 5-methylcytosine deglycosylase genes. Briefly, the target gene sequences of the mutants will be amplified by PCR, heat denatured and re-annealed for heteroduplex formation between mutated and wild-type DNA. Heteroduplexes will be identified through cleavage with *Cel*I endonuclease and can be visualized in a high throughput manner by size separation from the full-length PCR product on a polyacrylamide gel.

The mutants identified will be analyzed for their inhibition of gliadin and LMW glutenin synthesis. In case the deglycosylase genes of all three genomes of hexaploid and both genes of tetraploid wheat are actively transcribed and translated, separate mutants will have to be identified and combined by crossing. In order to speed propagation and characterization of the TILLED wheat mutants they are multiplied by doubled-haploid production via induced microspore embryogenesis permitting the production of ~1500 homozygous plants from a single spike in eight months [27]. Analyses of protein fractions of the mutants will be carried out with T-cell proliferation assays using the T-cell clones derived from celiac lesions [28]. The freeze-dried, proteolyzed endosperm protein samples will be dissolved in PBS with 2 mM $CaCl_2$ and treated with 100μg/ml human recombinant tissue transglutaminase for two hours at 370°C. The treated endosperm digests will be incubated overnight in triplicates in U-bottomed 96-well plates with 75,000 cells/well of DQ2 homozygous, irradiated (75-Gy) Epstein-Barr virus-transformed

B-lymphoblastoid cell lines in a volume of 100 µl of RPMI 1640 medium containing 10% pooled, heat inactivated human serum. These cell lines produce the DQ2 leucocyte antigen, to which the prolamin epitopes bind. After the incubation, 50 µl of freshly thawed T cells ($1x10^6$ cells/ml) will be added to each well. The proliferation of the T cells is evaluated by [3H] thymidine incorporation from 48 to 72 hours after addition of the T cells.

Alternatively it is intended to inactivate the wheat 5-methylcytosine deglycosylase genes specifically in the wheat endosperm by silencing hairpin RNA with a modified pHELLSGATE vector ([29,] Acc. No. AJ311874). Transformation will be carried out by co-cultivation of immature zygotic embryos with *Agrobacterium* [30, 31]. For specificity of silencing a HMW glutenin promoter and a nuclear localization signal can be employed.

ACKNOWLEDGEMENTS

Financial support by NIH grant 1R01 GM080749-01A2 and a Mercator Professorship from the German Research Foundation is gratefully acknowledged.

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Maize Mutant *Opaque2* and the Improvement of Protein Quality Through Conventional and Molecular Approaches

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Abstract

Maize endosperm protein is deficient in two essential amino acids, lysine and tryptophan. Several spontaneous and induced mutations that affect amino acid composition in maize have been discovered, amongst which the *opaque2* gene has been used in association with endosperm and amino acid modifier genes for developing quality protein maize (QPM), which contains almost double the amount of lysine and tryptophan compared to normal maize. These increases have been shown to have dramatic impacts on human and animal nutrition, growth and performance. A range of hard endosperm QPM germplasm has been developed at the International Maize and Wheat Improvement Center (CIMMYT) mostly through conventional breeding approaches to meet the requirements of various maize growing regions across the world. Microsatellite and SNP markers located within the *opaque2* gene provide opportunities for accelerating the pace of QPM conversion programmes through marker-assisted selection (MAS). Thus, CIMMYT scientists are developing a package of reliable, easy-to-use markers for endosperm hardness and free amino acid content in the maize endosperm. Recent technological developments in molecular biology at CIMMYT such as single seed-based DNA extraction and low cost, high throughput SNP genotyping strategies promise enhanced efficiency and cost-effectiveness of QPM molecular breeding programmes. Here we present a summary of QPM research and breeding with respect to the history of conventional improvement methodologies, genetic and molecular basis of *opaque2*, epistasis between *opaque2* and other high lysine mutant genes and recent advances in genomics technologies that could potentially enhance the efficiency of QPM molecular breeding in future.

Introduction

Maize (*Zea mays* L.) plays a very important role in human and animal nutrition worldwide. In the mature maize kernel, two principal components, the endosperm and the germ (embryo), contain most of the kernel protein. Generally, the endosperm accounts for 80-85% and the embryo accounts for about 8-10% of the total kernel dry weight [1]. The endosperm may contribute as much as 80% of the total kernel protein. While the germ protein is superior in quality, the endosperm protein suffers from poor quality with respect to human and animal nutritional needs. The major drawbacks of maize endosperm protein are i) its deficiency in two essential amino acids – lysine and tryptophan, ii) high leucine – isoleucine ratio and iii) low biological value of utilizable nitrogen. The need to genetically ameliorate the poor nutritional value of maize has been recognized for a long time and several mutations, both spontaneous and induced, have been identified that affect the amino acid composition of maize endosperm. Among them, a spontaneous mutation of maize with soft, opaque grains, named *opaque2 (o2)*, has been most intensively studied. Maize homozygous for the recessive *o2* allele has substantially higher lysine (>69%) and tryptophan content compared to normal maize [2].

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Maize Protein

Maize endosperm protein is comprised of different fractions. Based on their solubility, these can be classified into albumins (water-soluble), globulins (soluble in saline solution), zein or prolamine (soluble in alcohol) and glutelins (soluble in alkali). In normal maize endosperm, the average proportions of various fractions of protein are albumins 3%, globulin 3%, zein (prolamine) 60% and glutelin 34%, while the embryo protein is dominated by albumins (+60%), which are superior in terms of nutritional quality. The zein in maize endosperm is low in lysine content (0.1g/100g of protein), which negatively affects growth of animals [3]. In *opaque2* maize, the zein fraction is markedly reduced, by roughly 50%, with a concomitant increase in the relative amounts of nutritionally superior fractions such as albumins, globulins and glutelins. The endosperm of *opaque2* maize contains twice as much lysine and tryptophan and 30% less leucine than normal maize. The decreased level of zein (5-27%) in *opaque2* maize along with reduced leucine, leads to more tryptophan for niacin synthesis, helps to combat pellagra and significantly improves its nutritional quality.

High lysine mutants in maize

Several mutants have been detected that favorably influence maize endosperm protein quality by elevating levels of two essential amino acids, lysine and tryptophan. The discovery of *opaque2* [2] was followed by recognition of the biochemical effects of *floury2* (*fl2*) [4]. Searches for new mutants continued and resulted in the discovery of several others such as *opaque7* (*o7*) [5], *opaque6* (*o6*) [6], *floury3* (*fl3*) [6], *mucranate* (*Mc*) [7] and *defective endosperm* (*De-B30*) [8]. Attempts were also made to find genotypes with high lysine genes that retained a high level of zein fraction. Two such mutants, *opaque7749* and *opaque7455* (*o11*) [9] are particularly interesting as they have markedly higher levels of lysine as well as a high prolamine fraction. The specific chromosomal location is known for some of the mutants. For example, the *o2* mutant is located on chromosome 7, *fl2* on chromosome 4, *o7* on chromosome 10, *fl3* on chromosome 8 and *de-B30* on chromosome 7. The genetic action of some of the mutants is also known, for example, *o2, o6, o7* and *o11* are completely recessive. The two floury mutants are semi-dominant and exhibit variable expression for kernel opacity and protein quality depending on the presence of one or more recessives in the triploid endosperm. The mutant *De-B30* is dominant and shows dosage effects on kernel opacity and zein content [10].

Pleiotropic and secondary effects of *opaque2* and other high lysine mutants

Genes and gene combinations that bring about drastic alterations in either plant or kernel characteristics also produce several secondary or undesirable effects. The low prolamine and high lysine mutants are no

exception. In addition to influencing several biochemical traits, they adversely affect a whole array of agronomic and kernel characteristics. The *o2* and other mutants adversely affect dry matter accumulation resulting in lower grain yield due to increased endosperm size. The kernels dry slowly following physiological maturity of the grain and have a higher incidence of ear rots. Other changes generally associated with high lysine mutants include thicker pericarp, larger germ size, reduced cob weight, increased color intensity in yellow maize grains, and reduction in kernel weight and density. Thus, despite the nutritional superiority of *opaque2* maize, it did not become popular with farmers or consumers mainly because of reduced grain yield, chalky and dull kernel appearance and susceptibility to ear rots and stored grain pests. Hence, CIMMYT undertook to improve the phenotype of *opaque2* kernels to facilitate greater acceptability by developing hard endosperm grain types with the protein quality of chalky *opaque2* strains. CIMMYT received financial support, beginning in 1965, from the United Nations Development Programme and introduced gene modifiers that changed the soft, starchy endosperm to a vitreous type preferred by farmers and consumers whilst retaining the elevated levels of lysine and tryptophan. CIMMYT has subsequently developed a range of hard endosperm *opaque2* genotypes with better protein quality through genetic selection, which are popularly known as quality protein maize (QPM). Today's QPM is essentially interchangeable with normal maize in both cultivation and agronomic characteristics as well as being competitive in terms of yield, lodging, disease and pest resistance, and moisture level, while retaining the superior lysine and tryptophan content. In 2005, QPM was planted on 695,200 hectares across 24 developing countries.

Conventional breeding approaches to develop QPM

There are various breeding options for developing hard endosperm – high-lysine maize that is competitive in agronomic performance and market acceptance, which are based on specific endosperm high lysine mutants or available donor materials. The past approaches involving normal maize breeding populations have centered on altering germ-endosperm ratio, selection for multiple aleurone layers, and recurrent selection to exploit natural variation for high lysine content. Altering the germ-endosperm ratio to favor selection of larger germ size will have the dual advantage of increasing both protein quantity and quality [11] but it is not practical to attain lysine levels approaching those of *opaque2* maize. Besides, increased germ size has the disadvantage of contributing to poor shelf life of maize. Recurrent selection for high lysine in normal endosperm breeding populations has been largely unsuccessful due to the narrow genetic variation and heavy dependence on laboratory facilities. Alternatively, high-lysine endosperm mutants provided two attractive options: i) exploiting double mutants involving *o2*, and ii) simultaneous use of the *o2* gene with endosperm and amino acid modifier genes. In most instances, double mutant combinations involving *o2* and other mutants associated with endosperm quality were not vitreous [12]. The most successful and rewarding option exploited the combined use of *o2* with associated endosperm and amino acid modifier genes.

Segregation and analysis of kernels with a range of endosperm modification began at CIMMYT as early as in 1969 by John Lonnquist and V.L. Asnani. Initial efforts towards development of QPM donor stocks with good kernel phenotypes, as well as good protein quality, proved to be highly challenging. Two effective approaches, i.e., intra-population selection for genetic modifiers in *o2* backgrounds exhibiting a higher frequency of modified *o2* kernels and recombination of superior hard endosperm *o2* families, resulted in development of good quality QPM donor stocks with a high degree of endosperm modification. This was followed by the large-scale development of QPM germplasm with a wide range of genetic backgrounds, representing tropical, subtropical and highland maize germplasm and involving different maturities, grain color and texture. A summary of characteristics of promising QPM genepools and populations developed at CIMMYT is provided in **Table 1**. An innovative breeding procedure designated as 'modified backcross cum recurrent selection' was designed to enable rapid and efficient conversion programmes [13]. More recently, pedigree backcrossing schemes have been used to convert elite QPM lines to maize streak virus (MSV) resistance for deployment in Africa as well as conversion of elite African lines to QPM.

Table 1. Characteristics of QPM genepools and populations developed at CIMMYT (using *o2* and associated modifiers) including protein, tryptophan and lysine contents in the whole grain [12].

QPM Pop/Pool	Adaptation	Maturity	Color	Texture	Protein (%)	Trypto-phan in protein (%)	Lysine in protein (%)	Quality Index
Population 61	Tropical	Early	Y	Flint	9.2	0.98	4.2	3.8
Population 62	Tropical	Late	W	Semi-flint	9.9	0.92	3.9	4.4
Population 63	Tropical	Late	W	Dent	9.1	0.97	4.3	4.3
Population 64	Tropical	Late	W	Dent	9.6	1.00	3.8	4.3
Population 65	Tropical	Late	Y	Flint	9.2	0.96	4.2	4.4
Population 66	Tropical	Late	Y	Dent	9.3	1.01	4.3	4.3
Population 67	Subtropical	Medium	W	Flint	9.9	1.04	3.9	4.8
Population 68	Subtropical	Medium	W	Dent	9.5	1.01	4.0	4.3
Population 69	Subtropical	Medium	Y	Flint	10.0	0.98	4.2	4.4
Population 70	Subtropical	Medium	Y	Dent	9.3	1.10	4.3	4.7
Pool 15 QPM	Tropical	Early	W	Flint-Dent	9.1	0.94	4.2	4.6
Pool 17 QPM	Tropical	Early	Y	Flint	8.9	1.04	4.5	4.5
Pool 18 QPM	Tropical	Early	Y	Dent	9.9	0.93	4.0	4.6
Pool 23 QPM	Tropical	Late	W	Flint	9.1	1.03	3.8	4.2
Pool 24 QPM	Tropical	Late	W	Dent	9.4	0.92	3.8	4.0
Pool 25 QPM	Tropical	Late	Y	Flint	9.8	0.94	4.0	4.0
Pool 26 QPM	Tropical	Late	Y	Dent	9.5	0.90	4.1	4.3
Pool 27 QPM	Subtropical	Early	W	Flint-Dent	9.5	1.05	4.2	4.8
Pool 29 QPM	Subtropical	Early	Y	Flint-Dent	9.2	1.06	4.3	4.8
Pool 31 QPM	Subtropical	Medium	W	Flint	10.2	0.96	4.1	4.5
Pool 32 QPM	Subtropical	Medium	W	Dent	8.9	1.04	4.2	4.5
Pool 33 QPM	Subtropical	Medium	Y	Flint	9.3	1.05	-	4.2
Pool 34 QPM	Subtropical	Medium	Y	Dent	9.1	1.10	4.1	4.5

A QPM hybrid breeding programme was initiated at CIMMYT in 1985 as the QPM hybrid product has several advantages over open pollinated QPM cultivars: a) higher yield potential comparable to the best normal hybrids, b) assured seed purity, c) more uniform and stable endosperm modification, and d) less monitoring of protein quality required during seed production. Several QPM hybrid combinations were derived and tested through international trial series at multiple CIMMYT and NARS locations in Asia, Africa and Latin America. Current QPM breeding strategies at CIMMYT focus on pedigree breeding, whereby the best performing inbred lines and open pollinated varieties (OPV) with complementary traits are crossed to establish new segregating families. Both QPM×QPM and QPM × Normal crosses are made depending upon the specific requirements of the breeding project. In addition, backcross conversion is also followed to develop QPM versions of parental lines of popular hybrid cultivars that are widely grown in CIMMYT's target regions. Inbred lines developed through this process are then used in formation of QPM hybrids and QPM synthetic OPV [14].

Molecular basis of *o2* and modifier gene action

The breeding of QPM involves manipulation of three distinct genetic systems [14, 15]: i) the recessive mutant allele of the *o2* gene, ii) the

endosperm hardness modifier genes, and iii) the amino acid modifiers/genes influencing free amino acid content in the endosperm. The *o2* gene encodes a leucine-zipper class transcription factor that regulates the expression of zein genes and a gene encoding a ribosomal inactivating protein [16, 17, 18]. The homozygous recessive allele causes a decrease of the production of these zeins resulting in a corresponding increase in non-zein proteins, rich in lysine and tryptophan [19]. Additionally the recessive allele of the *o2* transcription factor also reduces the production of the enzyme, lysine keto-glutarate reductase, involved in free lysine degradation resulting in enhanced free lysine in the endosperm of *opaque2* maize. In the segregating generations, this recessive allele is selected either visually (identifying mosaic ears on F_2 harvests) or using molecular markers. The endosperm hardness modifier genes, which convert the soft/opaque endosperm to a hard/vitreous endosperm without much loss of protein quality, are selected through a low cost but effective method of light box screening, where light is projected through the vitreous grains or blocked by the opaque grains. Research at CIMMYT and elsewhere has demonstrated the quantitative and additive nature of the endosperm hardness modifying system [12]. Despite the presence of *o2* and associated endosperm hardness modifier genes, the lysine and tryptophan levels in segregating families vary widely indicating the existence of a third set of genes that modify the amino acid content, which necessitates systematic biochemical evaluation of lysine and/or tryptophan levels in each breeding generation.

Molecular breeding for QPM

The *opaque2* gene is recessive and the modifiers are polygenic. Their introgression into elite inbred lines is not straight forward because of three major factors: i) each conventional backcross generation needs to be selfed to identify the *opaque2* recessive gene and a minimum of four to six backcross generations are required to recover satisfactory levels of the recurrent parent genome, ii) in addition to maintaining the homozygous *opaque2* gene, multiple endosperm modifiers must also be selected, and iii) rigorous biochemical tests to ensure enhanced lysine and tryptophan levels in the selected materials in each breeding generation require enormous labor, time and financial resources. Although conventional breeding procedures have been used to convert commercial lines to QPM forms, these procedures are tedious and time consuming. Rapid advances in genomics research and technologies has led to the use of MAS which holds promise in enhancing selection efficiency and expediting the development of new cultivars with higher yield potential [20, 21]. While marker-assisted foreground selection [22, 23] helps in identifying the gene of interest without extensive phenotypic assays, marker-assisted background selection [24, 25, 26, 27] significantly expedites the rate of genetic gain/recovery of recurrent parent genome in a backcross breeding programme. With the development and access to reliable PCR-based allele-specific markers such as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs), MAS is becoming an attractive option, particularly for oligogenic traits such as QPM [28].

A rapid line conversion strategy for QPM has been developed [29], consisting of a two-generation backcross (BC) programme that employs foreground selection for the *opaque2* gene, in both BC generations, background selection at non-target loci in the BC_2 generation, and phenotypic selection for kernel modification and other desirable agronomic traits in two subsequent selfed generations. The rapid line conversion strategy outlined in this investigation brings together the salient features of both marker-assisted and phenotypic-based selection approaches such as fixing the large segregating generation for the target locus (*o2*), reducing the linkage drag by selection of flanking markers for recipient allele type, recovering maximum amount of recurrent parent genome within two BC generations and providing scope for precise phenotypic selection for desirable agronomic and biochemical traits on a reduced number of progeny.

Low-cost marker for *o2* and reliable markers for modifier genes of QPM

SSR markers (*umc1066, phi057 and phi112*) located within the *opaque2* gene provide an excellent foundation for MAS, but this alone is not sufficient to bring to bear the full effectiveness of molecular breeding for QPM genotypes. Each of the microsatellite markers located within the *o2* gene is associated with factors that challenge their routine use in MAS programmes. umc1066 is easily visualized on agarose gels but is commonly not polymorphic in CIMMYT breeding populations; *phi057* is difficult to visualize on agarose gels, usually requiring the use of polyacrylamide gels; *phi112* is a dominant marker, and hence cannot be used in the identification of heterozygotes in F_2/BC populations. However, *phi112*, which is based on a deletion in the promoter region, has the advantage of being a widely conserved marker, consistent with the phenotype in QPM germplasm tested. In order to overcome these difficulties, we have identified functional and more discriminative SNP markers that could be used in high throughput genotyping systems for selection of the *opaque2* genotype. These SNP markers have been used in the development of a medium throughput dotblot assay based on a detection system using hybridization on membranes, although these markers could also be readily detected using capillary electrophoresis systems.

Effective markers associated with modifying loci for both endosperm hardness and amino acid levels need to be identified. Unfortunately, little is known about the number, chromosomal location and mechanism of action of these modifier genes. A complex system of genetic control of these modifier loci with dosage effects, cytoplasmic effects, incomplete and unstable penetrance in different QPM germplasm creates a major bottleneck to the accelerated development of QPM germplasm. Using a limited set of restriction fragment length polymorphism (RFLP) markers and bulked segregant analysis [30], two chromosomal regions on the long arm of chromosome 7 that are associated with *o2* endosperm hardness modification were identified. The locus near the centromere is linked with the gene encoding the 27 kDa gamma zein. More recently, the analysis of two different QPM genotypes, K0326Y and cm105Mo2 (derived from CIMMYT's Pool 33 QPM), corroborated the existence of a common quantitative trait locus (QTL) near the centromere of chromosome 7 that appears to have a major effect (30% of the phenotypic variance) on *o2* endosperm modification, in addition to a QTL on 9.04/9.05 [31]. In a specific F_2 population segregating for kernel vitreousness, these two loci accounted for 40% of the phenotypic variation and thus may prove to be strong candidates for MAS for QPM breeding.

Precise information on genes controlling the level of amino acid modification, especially with respect to lysine and tryptophan, is relatively scarce and studies to date have found several quantitative trait loci (QTL) on many of the maize chromosomes [32, 33]. The free amino acid (FAA) content in Oh545o2 is 12 times greater than its wild-type counterpart, and three and 10 times greater than in Oh51Ao2 and W64Ao2, respectively. QTL mapping involving these materials identified four significant loci that account for about 46% of the phenotypic variance for FAA [34]. One locus on the long arm of chromosome 2 is coincident with genes encoding a *monofunctional aspartate kinase 2* (*Ask2*), whereas another locus on the short arm of chromosome 3 is linked with a *cytosolic triose phosphate isomerase 4*. Subsequent feedback inhibition analysis has suggested that *Ask2* is the candidate gene associated with the QTL on 2S [35] and that a single amino acid substitution in the C-terminal region of the *Ask2* allele of Oh545o2 is responsible for altered basal activity of the enzyme [36]. Using a RIL population from the cross between B73o2 (an *o2* conversion of B73) and a QPM line (CML161), it was possible to identify three QTL for lysine content and six QTL for tryptophan content, which explained 32.9% and 49.1% of the observed variation, respectively [37]. Thus a series of molecular markers (**Table 2**) for manipulation of different genetic components of QPM is available,

and hence their validation and fine mapping in appropriate breeding populations should now be carried out in order to establish a single cost effective MAS assay for molecular breeding of QPM. Concerted research efforts to quantify the effect of these loci affecting endosperm hardness and amino acid levels, coupled with marker development and validation will also accelerate the pace and precision of QPM development programmes.

Table 2. Molecular markers currently being validated at CIMMYT for manipulation of different genetic components of QPM.

Chromosome	Flanking markers	LRS*	PEV**	Reference
1) *opaque2* **gene**				
7S	umc1066, phi057, phi112 (located within the o2 gene)			[29,38]
2) Endosperm harness modification (based on K0326YQPM × W64Ao2)				
1.05-1.06	umc1076-umc1335	12.8	5	[31]
7.02	umc1978-bnlg1022 (27kDa gamma zein gene)	75.3	28	[31]
9.04-9.05	umc1771-umc1231	29.4	12	[31]
10.02	phi063-umc1432	16.7	11	[31]
3) Free amino acid content (FAA)				
2L	bmc1633-bmc1329 (aspartate kinase2)	14.8	11	[34,36]
2S	bmc1537-bmc2248	12.8	10	[33]
3S	bmc1904-bmc2136/bmc1452	17.9	15	[33]
7L	bmc2328b-phi045	12.6	10	[33]

* LRS is the likelihood ratio statistic that measures the significance of the QTL.
** PEV (percent explained variance) is the percent of total variance explained by the QTL.

Seed DNA-based genotyping and MAS for QPM

Leaf collection from the field, labeling and tracking back to the source plants after genotyping are rate-limiting steps in leaf DNA-based genotyping. Recently, an optimized genotyping method using endosperm DNA sampled from single maize seeds was developed at CIMMYT [39], which has the potential to replace leaf DNA-based genotyping for marker-assisted QPM breeding. This method is suitable for various types of maize seeds, produces high quality and quantity of DNA and has minimal effects on subsequent germination and establishment. A substantial advantage of this approach is that it can be used to select desirable genotypes before planting, which can bring about dramatic enhancements in efficiency by planting only the plants containing the *o2* gene in recessive form in BC_nF_2 generations of Normal × QPM crosses, and also by minimizing the labor costs and scoring error associated with light-box screening of a large number of grains for endosperm hardness. Over several breeding cycles, this is likely to lead to cumulative and accelerated gains in selection pressure (such as light box screening for endosperm hardness modification and systematic biochemical evaluation of lysine and/or tryptophan) and improvements in overall QPM breeding efficiency.

Integrated approaches for improvement of enhanced protein quality in maize

Research at CIMMYT is currently focused on developing a package of molecular markers for cost-effective large scale, marker-assisted QPM breeding programme. We have developed gene-based SNP markers and a medium throughput, low cost dot blot genotyping assay using a membrane-based hybridization system for the *opaque2* gene. This system is likely to be especially useful for NARS programmes with limited lab facilities. In addition, intensive efforts are being made to develop and validate new as well as existing markers for the endosperm and amino acid modifier genes across a wide range of populations and improved pools. A new breeding programme has recently been initiated at CIMMYT in collaboration with Guizhou Academy of Agricultural Sciences, China, to pyramid the *o2* with another high lysine mutant, *o16*, which was selected from Robertson's Mutator (Mu) stock where the lysine content in the F_3 *o2o2o16o16* families derived from recombination of both *o2* and *o16*, was about 30% higher than that of *o2o2* or *o16o16* F_3 families [40]. Using both genes together could lessen the requirement for phenotypic screening for amino acid content and genetic screening for amino acid modifiers.

Recent efforts in genetic transformation are focused on developing a dominant *opaque2* trait in maize. RNA interference (RNAi) technology has been used to reduce 22-kDa [41] and 19-kDa alpha zeins [42, 43] using antisense transformation constructs, which result in moderate increases (15-20%) in lysine content. In a recent study, using an improved double strand RNA (dsRNA) suppression construct, Huang, *et al.* [44] reported lysine and tryptophan levels similar to conventionally bred QPM genotypes. While the dominant nature of the anti-sense transgene is a definite advantage compared to recessive allele of *o2*, the opaque endosperm still needs to be modified by endosperm modifier genes whose epistasis with the transgene has not yet been tested. In addition, social acceptance and legislative concerns regarding genetically modified crops may prove to be a major bottleneck to their practical utility and large scale adoption in some developing countries. Efforts are underway at CIMMYT-Africa to create transgenic events using RNAi that could potentially enhance lysine and tryptophan to levels similar to QPM. Identification and improvement of lines with enhanced regenerability and transformability is in progress to facilitate RNAi efforts. Tropical maize lines, CML390, CML442, CML254 and CML492 were identified for their better regenerability and transformability, while CML395 was improved for transformation efficiency by crossing to temperate inbred line A188.

Cost effectiveness of marker assisted QPM breeding programmes

Detailed cost-benefit analysis carried out at CIMMYT [45] with respect to MAS for QPM (*o2* gene alone) suggested that the relative cost effectiveness would depend upon specific circumstances. In cases where segregating materials can be visually inspected or light box screened to identify *o2* containing ears, conventional methods may prove to be more cost-effective although less accurate, while MAS may be a valuable tool with certain genetic backgrounds which do not allow easy phenotypic detection of *o2*. More recently, four BC_1F_2 populations segregating for *o2* were used to test the error rate of light-box screening and to estimate the cost of genotyping using a seed DNA-based genotyping method recently developed at CIMMYT, which makes it possible for MAS to be carried out before planting. For two populations where the light-box error rate was over 30%, it is predicted that there is an overall benefit from using MAS. Effective use of molecular markers for QPM would be achieved through seed DNA-based genotyping, and use of flanking markers around the *o2* gene to improve the efficiency of backcross selection. In addition, simultaneous MAS for the *o2* gene as well as modifier genes for amino acid content and endosperm hardness would provide a much more compelling cost-benefit ratio. This would reduce the need for phenotypic screening, saving time and screening costs. Moreover, when MAS is implemented for simultaneous selection of endosperm modifiers and other multiple traits such as disease resistance and other quality traits, the added value of MAS will be cumulative in terms of cost and time efficiency, as well as selective gain.

Conclusion

Biofortification of maize grains is an important area of research for which *opaque2* provides an ideal platform upon which a number of nutritionally important traits such as enhanced iron and zinc content and low phytate content (for increased bioavailability of nutrients) could be

combined for multiple benefits. Considering the pace of technological developments in genome research, a molecular breeding option is likely to be the leading choice in the future for stacking a range of nutritionally important specialty traits, especially those governed by recessive genes. With respect to *opaque2*, an additional challenge to field implementation of QPM is its recessive nature [14]. If QPM is pollinated by normal maize pollen, there may be loss of high protein quality resulting in erosion of the trait in farmer saved seed systems. Though several years of QPM testing at CIMMYT and elsewhere has proved this apprehension to be not significantly valid, training on good seed production practices to the local communities may ensure sustainable higher nutritional benefits of QPM in the long term.

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Mutation Breeding for Oil Quality Improvement in Sunflower

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Abstract

Although sunflower oil is appreciated as a high quality commodity, new emerging markets and increasing concern about health risks are demanding changes in oil quality. The optimal quality of oils depends on their intended use either for food or non-food applications. The fatty acid composition and the total content and profile of tocopherols have been the most important traits considered in breeding for oil quality. Applications demanding a high nutritional value (salad oil) require a reduction of saturated fatty acids and enhancement of the vitamin E (alpha-tocopherol) content of the oil. Uses of oils in the food industry requiring plastic fats (margarines) demand an increased concentration of saturated fatty acids to avoid hydrogenation. High temperature processes (frying, biolubricants) need oils highly resistant to thermo-oxidation, with a high concentration of oleic acid and antioxidants (gamma- and delta-tocopherol). In sunflower, the utilization of mutagenesis has been the most successful procedure to generate genetic variability for these quality components. The mutagenic treatment is usually applied to seeds to obtain the M_1 generation and mutants are detected analyzing M_2 half seeds, allowing identification of mutants in one year. The most valuable sunflower oil quality mutants produced have been those with high oleic acid (>80%), high levels of either palmitic or stearic acid (>25%), low total saturated fatty acids (<7%) and increased levels of beta- (>65%), gamma- (>95%), and delta-tocopherol (>45%). The novel traits are, in all cases, governed by a small number of genes, which facilitate their management in plant breeding. This induced variation opens up the possibility of tailoring specialty sunflower oils for specific food and nonfood applications.

Introduction

Today, sunflower (*Helianthus annuus* L.) oil is the fourth most important vegetable oil in world trade, after soybean, palm, and canola, with an annual production around nine million tons and cultivated acreage over 22 million ha [1]. Sunflower oil has been traditionally appreciated as a high-quality commodity in the world oil market. However, new emerging markets together with an increasing concern about health risks of foods are demanding changes in oil quality. The optimal quality of sunflower oil depends on the intended use of the oil, either for food or non-food applications. The former include salad and cooking oils as well as oils for the food industry (margarines, shortenings, etc.). The latter comprises countless industrial sectors such as biodiesel and lubricants, surfactants, surface coatings, cosmetics, etc. Consequently, selection for a broad spectrum of oil types is required to fulfill the present and future needs of the industry. In general, oil characteristics that are undesirable for a particular application are required for others. Therefore, breeding for improved oil quality is a continuous exercise of divergent selection.

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Components of oil quality: Breeding objectives for oil quality in sunflower

Vegetable oils mainly contain molecules of triacylglycerol (TAG). Triacylglycerols are glycerol molecules containing one fatty acid esterified to each of the three hydroxyl groups. The stereochemical positions of the three fatty acids in the glycerol molecule are designed *sn-1*, *sn-2*, and *sn-3* (**Fig. 1**).

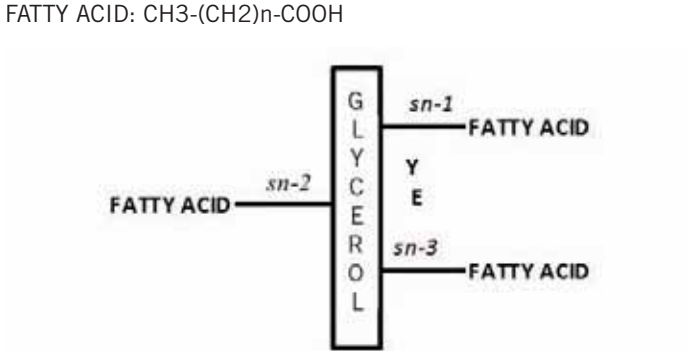

Figure 1 Schematic structure of a triacylglycerol molecule; *sn-1, sn-2* and *sn-3* refer to the carbon numbers of the glycerol; *sn-2* is a chiral center.

In sunflower, triacylglycerols represent more than 95% of the total oil weight. The remainder are lipid and lipid-soluble compounds, some of them of great value because of the functional and nutritional properties they confer to the oil. The fatty acid composition of the oil and the distribution pattern of the fatty acids within the triacylglycerol molecule determine the physical, chemical, physiological and nutritional properties of vegetable oils [2]. Therefore, breeding for oil quality in sunflower has mainly focused on the modification of the relative amount of fatty acids that are present in the oil, but in recent years minor compounds with important nutritional and antioxidant value, especially tocopherols and phytosterols, have also attracted the attention of sunflower breeders.

Fatty acids differ in their number of carbon atoms and/or number and position in the carbon chain of double bonds. Depending on the presence or absence of double bonds in the fatty acid chain, the fatty acids are divided into saturated, which do not contain double bonds, monoinsaturated, with one double bond and polyunsaturated, which contain more than one double bond. For example, 18:1 designates an 18-carbon monounsaturated fatty acid (oleic acid). The unsaturated fatty acids can also have two possible configurations, *cis* or *trans*, depending on the relative position of the alkyl groups. Most naturally occurring unsaturated fatty acids have the *cis* orientation, although several common industrial processes such as hydrogenation, generate *trans* isomers which are considered to be nutritionally undesirable [3]. The tocopherols are a group of four lipid-soluble substances with molecular structure comprised of a chromanol ring and a saturated phytyl side chain (**Fig. 2**). The four tocopherols, named alpha-, beta-, gamma-, and delta-tocopherol, differ

Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009,**177-181**

in the number of methyl substituents and the pattern of substitution in the chromanol ring [4].

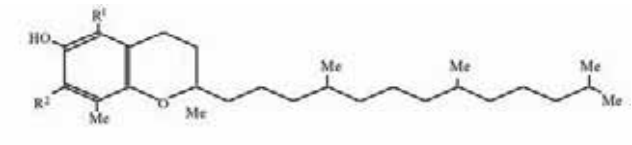

Figure 2 Chemical structure of tocopherols. Me=methyl groups. R^1=Me; R^2=Me: α-tocopherol; R^1=Me; R^2=H: β-tocopherol; R^1=H; R^2=Me: γ-tocopherol; R^1=H; R^2=H: δ-tocopherol.

Nutritional and functional properties of the oils are largely determined by the fatty acid composition of the oil, the distribution pattern of the fatty acids within the triacylglycerol molecule, and the total content and composition of natural antioxidants especially tocopherols. The main fatty acids in sunflower oil and their outstanding properties are given in **Table 1**.

Table 1. The major fatty acids in sunflower oil and their outstanding properties.

Trivial name	Symbol	Nutricional properties	Physical properties	Oxidative stability	Standard content (%)	Richest source (%)	Lowest source (%)
Palmitic	16:0	Hipercholesterolemic	Solid	High	6	33	4
Stearic	18:0	Neutral	Solid	High	5	37	2
Oleic	18:1	Hypocholesterolemic	Liquid	High	21	94	5
Linoleic	18:2[a]	Hypocholesterolemic	Liquid	Low	68	85	2

[a] Essential fatty acid that is not synthesized by the human body and has to be obtained from the diet.

Standard sunflower oil has an average fatty acid composition of 11% saturated fatty acids (palmitic-16:0- and stearic acid-18:0-), 20% oleic acid (18:1), and 69% linoleic acid (18:2), although the ratio linoleic/oleic acid, which together account for about 90% of the total fatty acids, is environmentally dependent, with a range of variation from 0.8 to 5.0 depending mainly on the temperature during seed development. From a nutritional point of view, the effect of fats on cholesterol levels depends on their fatty acid composition [5]. In general, saturated fatty acids are regarded as undesirable for human consumption, as they have a hypercholesterolemic effect, increasing both serum total cholesterol and low-density lipoproteins (LDL), and therefore the ratio LDL/HDL (high-density lipoproteins), compared with mono- and polyunsaturated fatty acids, which act to lower serum cholesterol. The exception is stearic acid which, in spite of being saturated, does not have any effect on cholesterol levels [6]. Oleic and linoleic acids are hypocholesterolemic but, although linoleic acid is an essential fatty acid, oleic acid is nowadays considered as the preferred fatty acid for edible purposes, as it combines a hypocholesterolemic effect [7] and a high oxidative stability [8]. Another parameter playing an important role in lipid nutritional value is the stereochemical position of the fatty acids in the three positions of the triacylglycerol molecule, as the absorption rate of the fatty acids is higher when they are sterified at the central *sn-2* TAG position than when they are at the external *sn-1* and *sn-3* positions [9]. Thus, vegetable oils having undesirable saturated fatty acids at the *sn-2* position, such as palm oil or partially hydrogenated fats, are more atherogenic than those having a similar total saturated fatty acid content but distributed at the external positions, as is the case of high palmitic acid sunflower [10].

Tocopherols exhibit differential *in vivo* and *in vitro* antioxidant activities. Alpha-tocopherol exerts a maximum *in vivo* activity, also known as vitamin E activity, but poor *in vitro* protection of the extracted oil, whereas gamma-, delta- and to a lesser extent beta-tocopherol are powerful *in vitro* antioxidants with low vitamin E value [4]. Applications demanding a high nutritional value of the oil (e.g. salad oil) will require an enhancement of the vitamin E (=alpha-tocopherol) content of the oil. Other important oil quality components with important nutritional value, due to their ability to lower total and LDL serum cholesterol in humans, are phytosterols.

From a technological point of view, key aspects for most applications are plasticity and resistance to oxidation, particularly at high temperatures. Plastic fats are widely required in the food industry for the production of shortenings, margarines, and many specialty products. Because standard sunflower oil is mainly made up of unsaturated fatty acids, it is liquid at room temperature. Accordingly, its utilization by the food industry usually requires a previous chemical hardening to change it to a solid or semi-solid state, usually conducted by partial hydrogenation. However, partial hydrogenation also induces *cis-trans* isomerisation of fatty acids [3], resulting in the production of *trans*-fatty acids associated with heart disease [11]. These uses demand sunflower oils with high concentration of saturated fatty acids, preferably stearic acid which, as mentioned above, does not modify the plasma cholesterol content [6]. For high temperature applications and deep frying, oils with a lower content of polyunsaturated fatty acids are required. Thermo-oxidative experiments to test oil stability carried out at 180°C for 10-hour monitorizing the formation of polar and polymer compounds of different oils showed that TAG polymerization varied with the type of oil [12]. For example, commodity polyunsuturated oils such as soybean, canola and standard sunflower oils must be rejected after eight hours at 180°C, while high-oleic sunflower oil could still be used after 10 hours and the high-palmitic and high-oleic oil last even longer. This indicates that oils with a higher content of oleic and palmitic acids are the best for high temperature applications. Tocopherols could also modify the thermo-oxidative stability of the oils. Experiments including high-oleic, high-palmitic sunflower oils containing either α-tocopherol or γ-tocopherol showed that the oil with γ-tocopherol had a much better performance at high temperature, as it produced less than half the polymerised triacylglycerols than the same oil with α-tocopherol [13].

Following the previous description of components of quality, it is clear that breeding objectives for oil quality in sunflower are multiple depending on the intended use of the oil. For example, applications demanding a high nutritional value of the oil (e.g. salad oil) will require a reduction of saturated fatty acids and enhancement of the vitamin E (=alpha-tocopherol) content of the oil. Conversely, the use of sunflower oil in the food industry requiring solid or semi-solid fats (e.g. margarines and shortenings) will demand an increased concentration of saturated fatty acids, mostly stearic, in order to avoid hydrogenation. For other food applications, including high temperature processes (e.g. frying oil industry), sunflower oils with high concentration of oleic acid and the *in vitro* antioxidants gamma- and/or delta-tocopherols are much more appropriate. For industrial non-food uses requiring high oxidative stability at high temperatures (biodiesel and biolubricants) high or very high levels of oleic acid and gamma- and/or delta tocopherols are required.

Sources of variability and screening procedures

Breeding for oil quality requires the availability of sources of variation for the different quality components and adequate screening techniques to measure them. Mutagenesis has been used successfully to generate genetic variability for sunflower seed oil quality traits. One of the most valuable mutants obtained was the variety "Pervenets," with oleic acid content above 75%. It was produced at the All-Union Research Institute of Oil Crops of the former USSR, after treatment with dimethyl sulfate (DMS) [14]. Inbred lines have been derived from Pervenets with oleic acid contents higher than 90% [15]. High and mid oleic acid mutants were also developed using ethyl methanesulphonate (EMS) treatments [16]. High levels of stearic acid (>25%) were achieved using the chemical mutagens EMS and sodium azide (NaN_3) [17, 18] and high levels of

palmitic acid were obtained using both physical mutagens (X-rays and γ-rays) [17, 19, 20, 21] and EMS [22]. Alternatively, low levels of saturated fatty acids were also obtained using chemical mutagenesis (EMS and N-methyl-N-nitrosourea, NMU) [23]. Mutants with increased levels of gamma-tocopherol (>95%) have been isolated following chemical mutagenesis with EMS [24]. A detailed list of the induced mutants with improved oil quality developed in sunflower and mutagens used are presented in **Table 2**.

The mutagenic treatment is usually applied to the seeds, which after treatment are named M_1 seeds. Mutants can be detected in the M_2 generation. In sunflower, both the fatty acid and the tocopherol profile of the seeds are mainly under gametophytic control, i.e. they are governed by the genotype of the developing embryo. Therefore, mutants are detected by analyzing M_2 half-seeds.

Breeding programmes to improve seed oil quality traits require the development of accurate and fast screening techniques to measure them. Since fatty acid and the tocopherol profile of the sunflower seed oil are under gametophytic control, selection for these oil quality traits can be conducted at the single-seed level. Nondestructive methods to measure these traits in single seeds have been developed for different oil seed species. The half-seed technique developed for nondestructive analysis of the fatty acid composition of single seeds has been adapted to sun-

Table 2. Fatty acid and tocopherol composition of the principal induced mutants of sunflower in comparison with the standard types. The concentrations of the most altered fatty acids or tocopherols are printed in bold.

Induced mutants and derived lines with altered fatty acid composition

Mutant or line	Oil type	Fatty acid composition (%) [a]					Mutagenic treatment	Refe rence
		16:0	**16:1**	**18:0**	**18:1**	**18:2**		
Standard[b]	Low/medium 18:1	5.7	----	5.8	20.7	64.5		
		6.5	----	3.0	40.9	49.6	----	
Low content in saturated fatty acids								
LS-1	Low 18:0	5.6	----	**4.1**	20.2	67.4	NMU (4-8 gkg $^{-1}$)	[23]
LS-2	Low 18:0	8.6	----	**2.0**	10.8	75.0	NMU (4-8 gkg $^{-1}$)	[23]
LP-1	Low 16:0	**4.7**	----	5.4	23.8	63.7	EMS (4-8 gkg $^{-1}$)	[23]
High content in palmitic acid								
275HP	High 16:0	**25.1**	6.9	1.7	10.5	55.8	γ-rays (1550 R)	[19]
CAS-5	High 16:0	**25.2**	3.7	3.5	11.4	55.1	X-rays (150Gy)	[17]
CAS-12	High 16:0	**30.7**	7.6	2.1	**56.0**	3.1	X-rays (150Gy)	[20]
CAS-37	High 16:0-16:1	**29.5**	**12.3**	1.4	5.4	38.7	X-rays (150Gy)	[21]
NP-40	High 16:0	**23.9**	3.4	2.0	20.4	50.7	EMS (70 mM)	[22]
High content in stearic acid								
CAS-3	High 18:0	5.1	----	**26.0**	13.8	55.1	EMS (70 mM)	[17]
CAS-4	Medium 18:0	5.4	----	**11.3**	34.6	48.0	NaN_3 (2-4 mM)	[17]
CAS-8	Medium 18:0	5.8	----	**9.9**	20.4	63.8	NaN_3 (2-4 mM)	[17]
CAS-14	Very high 18:0	8.4	----	**37.3**	12.4	38.0	NaN_3 (2-4 mM)	[18]
High content in oleic acid								
Pervenets	High 18:1	----	----	----	**79.3**	14.8	DMS (0.5%)	[14]
HO lines[d]	High 18:1	4.9	----	2.9	**90.3**	1.8	DMS (0.5%)	[15]
M-4229	High 18:1	3.4	----	4.1	**86.1**	3.9	EMS (0.1%)	[16]
M-3067	Mid 18:1	3.9	----	5.2	**54.6**	33.9	EMS (0.1%)	[16]

Induced mutants and derived lines with altered tocopherol composition

Mutant or line	Oil type	Tocopherol composition (%)				Mutagenic treatment	Reference
		α-T	**β-T**	**γ-T**	**δ-T**		
Standard	High α-T	95.0	3.0	2.0	0.0		
IAST-1	High γ-T	5.0	----	**95.0**	----	EMS (70 mM)	[24]
IAST-540	High γ-T	5.0	----	**95.0**	----	EMS (70 mM)	[24]
IAST-4	High δ -T	4.0	3.0	34.0	**58.0**	Recombination[e]	[24]
IAST-5	High β -T	25.0	**75.0**	----	----	Recombination[e]	[24]

[a] 16:0=palmitic acid; 16:1= palmitoleic acid; 18:0=stearic acid; 18:1=oleic acid; 18:2=linoleic acid.
[b] Data of standard cultivars obtained in cool and warm environments, respectively.
[c] Contains also 4.6% of palmitolinoleic acid (16:2) and 5.8% of asclepic acid (18:1$^{\Delta 11}$).
[d] Mean of four high oleic acid lines incorporating the Pervenets mutation.
[e] Obtained through recombination between IAST-1 and the natural variant T589, with medium β-tocopherol content.

flower [25]. It consists of the removal of a small seed portion in the seed extreme distal to the embryo in such a way that the germination capacity of the seed is not affected. The excised half seed is used for chemical analysis, whereas the other half seed containing the embryo can be sown to produce a viable plant. The half-seed technique has been also used in sunflower for the nondestructive analysis of tocopherol composition and total tocopherol content [24].

Selection for seed quality at a single seed level has been facilitated by the use of near-infrared spectroscopy (NIRS) for analyzing the fatty acid profile of intact or hulled individual kernels. NIRS is a fast, non-destructive and cost-effective technique that permits the simultaneous analysis of multiple constituents in a single measurement. This requires the previous development of individual calibration equations for every constituent to transform NIRS spectral data into chemical information. However, the application of this technique to sunflower breeding requires the use of small samples of intact achenes. The feasibility of the use of NIRS in the analysis of oil quality components has been demonstrated for the determination oleic and linoleic acid concentration in the seed oil in intact achenes [26] or for large-scale screening for high stearic acid concentration in single hulled sunflower seeds [27].

Breeding and genetics

Comprehensive reviews on the genetics of quality traits in sunflower have been published [28, 29]. In general, the novel fatty acid and tocopherol traits obtained by mutagenesis are controlled by the genotype of the developing embryo and they are in all cases governed by a reduced number of genes, which considerably facilitates their management in plant breeding. For example, recessive alleles at three loci (*P1, P2, P3*) are involved in the control of high levels of palmitic acid content and partially-recessive alleles at loci *Es1, Es2* and *Es3* control high levels of stearic acid content. Detailed genetic studies showed that some of these recessive alleles were already present in the original lines and that the mutagenic treatments only induced a single recessive mutation in the wild dominant alleles, *P1* to *p1* in the high palmitic mutant CAS-5 and *Es1* to *es1* in the high stearic mutant CAS-3 [28]. The high oleic acid content is controlled by one principal gene *Ol1* and several modifier genes. Similarly, two unlinked genes, *Tph1* and *Tph2*, control altered tocopherol composition. Moreover, in recent years molecular markers have been developed for some of the traits, for example for high stearic and high oleic acid contents [30], or high beta- and gamma-tocopherol contents [31, 32]. The use of these molecular markers will contribute to improving breeding efficiency.

Concluding remarks

A tremendous range of variation for the main components of sunflower oil quality, the relative concentration of all the individual fatty acids and tocopherols present in sunflower seeds, has been obtained through mutagenesis. In contrast to other oilseed crops, this variation has been much higher than that obtained from naturally occurring variation. This progress has been made possible by the combination of chemical and physical mutagenesis and advances in analytical techniques which allow very rapid, cheap, reliable, and nondestructive analyses of fatty acid and tocopherols at a single-seed level. Mutants with high, intermediate and low levels of saturated fatty acids, mid and high levels of oleic acid, as well as high levels of beta-, gamma-, and delta-tocopherol have been developed providing more variability for fatty acid and tocopherol profiles in sunflower oil than in any other oilseed crop. For example, sunflower oil with low saturates and very high oleic acid, has the highest oleic acid levels (>92%) of any vegetable oil currently in the market. The novel fatty acids and tocopherol traits have a high environmental stability and they are in all cases governed by a small number of genes. Therefore, they can be easily managed in breeding programmes aimed at developing cultivars incorporating these traits. Moreover, progress has been made over the last few years in the development of molecular markers for some of the modified oil quality traits, which will contribute to improve breeding efficiency, especially for those traits controlled by recessive genes or those more affected by the environment. The combination of several quality traits in a single phenotype will enable tailoring specialty oils providing essentially "new oilseed crops" for specific uses in the food and non-food industry, thus guaranteeing a promising future to sunflower in the global world market. In addition to the two sunflower oils currently available, the standard low oleic and the high-oleic acid oil (the first variant obtained by mutagenesis), new sunflower oils with modified tocopherols and fatty acid composition, developed through combination of the available mutants are foreseeable in the next few years. Some relevant examples are:

- Low saturated oils, both in standard and high oleic acid backgrounds and rich in vitamin E, suitable for salads and cooking.
- High stearic acid oils, in standard and high oleic acid backgrounds and rich in vitamin E, suitable for the production of more healthy margarines.
- Mid and high oleic acid oils combined with high content of *in vitro* antioxidants (gamma- and delta-tocopherol) usable for biodiesel and other applications requiring high temperature processes (deep frying, biodegradable lubricants).
- High palmitic acids oils in high oleic background and rich in gamma or delta tocopherols as an alternative for high temperature processes.

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The Multiple Uses of Barley Endosperm Mutants in Plant Breeding for Quality and for Revealing Functionality in Nutrition and Food Technology

L Munck* **& B Møller Jespersen**

Abstract

This paper describes, with examples, how biochemical seed mutations can be used not only to improve quality in cereals, but also to understand functionality in nutrition and in food processing, and even to provide an overview of gene expression for improved genetic theory. A high lysine barley mutant Risø 1508 (*lys3.a*) cross breeding material developed at Carlsberg between 1973 and 1988 is used to demonstrate how Near Infrared Reflectance (NIR) spectra evaluated by a Principal Component analysis (PCA) score plot can visualize an improved chemical composition resulting from an altered genotype. By introducing high lysine *lys3.a* recombinants with increased starch and yield to pig feeding, protein concentration in the feed, nitrogen load on the animal and, also, importantly nitrogen load on environment could be reduced by 15-20% without compromising meat production. The decreased nitrogen load on the animal spares the energy that is necessary to catabolize the surplus of non-essential amino acids. The lower carbohydrate content and lower energy digestibility in the improved *lys3.a* lines is likely to be compensated by a more efficient metabolism of the protein. Thus, the high lysine mutants are contributing to the understanding of animal physiology. Similarly, the technological importance of slender cell walls and low ß-glucan was elucidated using mutant M-737 from Carlsberg, which accelerated malt modification to spare one to two days. The vast majority of scientists are traditionally rather pessimistic about the possibility of repairing the negative pleiotropic effects of mutations by engaging in the hard and time-consuming work of cross breeding. An open holistic exploratory strategy is necessary in order to evaluate the great complexity of the pleiotropic effects of a mutant gene that can not be forecasted by prior scientific knowledge. This is now possible by exploiting NIR Spectroscopy as a coarse overview of the phenotype (Phenome) on the level of chemical bonds.

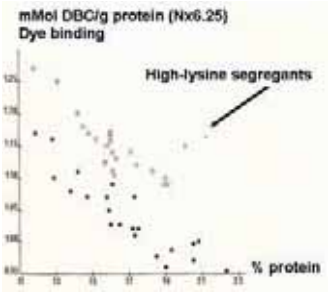

Figure 1 Selection of high lysine (squares) and normal (black dots) recombinants from a cross between Hiproly x normal barley from the dyebinding/Kjeldahl protein plot (Munck, 1972).

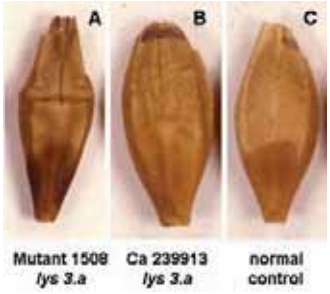

Figure 2 Visualising the result of 15 years of breeding at Carlsberg for improved seed quality, starch content and yield in Risø 1508 (lys3.a mutant). **(A)** Original M-1508 mutant. **(B)** Improved high-lysine recombinant Ca239913. **(C)** Normal control (Munck, 1988).

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Introduction

This paper focuses on what can be learnt, both practically and theoretically, from the intense research on cereal-seed mutants conducted in the 1960's through the 1980's sponsored by the IAEA/FAO. In the 1960's there was major concern regarding the low nutritional quality of cereal protein as a food to children, where lysine was the limiting amino acid. In 1964 a dye-binding capacity (DBC) screening method (**Fig. 1**) was developed to assay basic amino acids (lysine), which was used in 1966 to identify the first high lysine gene in an Ethiopian cultivar, later called "Hiproly," from the world barley collection (CI3947) [1]. Hans Doll and his group at the Risø National Nuclear Laboratory in Denmark used the DBC method extensively in the 1970's to isolate about 25 barley mutants with moderate-to-high lysine content in their protein. These were all more or less depleted in starch and had lower yields and seed quality than their mother variety [2]. The most radical mutant was Risø 1508 in Bomi (gene *lys3.a*), which had a 45% increase in lysine and a biological value near to milk proteins. A pig trial conducted by H.P. Mortensen and A. Madsen at the Royal Veterinary and Agricultural University (KVL) using Risø 1508 recorded an 84% increase in growth over three months (**Fig. 3**), reducing the barley consumed to obtain a 90 kg slaughter weight from 538 kg for normal barley to 209 kg for the Risø 1508 mutant. The experiment was made without protein additives.

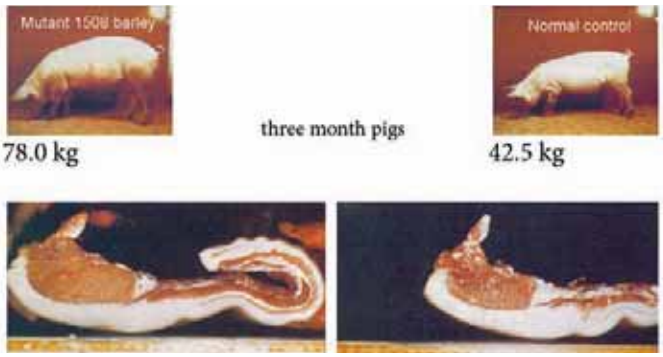

Figure 3 Functionality of improved protein by essential amino acids (lysine) demonstrated in a pig feeding trial with the Risø M-1508 (lys 3.a) mutant and a normal control without protein supplement. Mortensen and Madsen, 1988. See **Table 3**.

In 1973, the senior author started a programme at the Carlsberg Research Laboratory to induce mutants in barley with reduced extract viscosity (ß-glucan) and to breed for a genetic background for the *lys3a* gene that could improve seed quality (**Fig. 2**), starch content and yield. This was successful (**Fig. 3**, **Table 1**, [3]). However, while protein (soy bean meal) was imported to the EU at world market prices, starch obtained a higher market price through earlier EU subventions on cereals. Starch was three to six absolute percent lower in high-lysine barley. This was one of the reasons why the high protein-lysine mutant is today almost forgotten. However, the current rapidly rising food prices may introduce new world market priorities that support improved cereal protein in barley, maize and sorghum.

Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **182-186**

Table 1. Agronomic characters of high-lysine lys3.a breeding lines, mutant 1508 (lys3a) and Bomi in relation to national barley reference (from Bang-Olsen, *et al.*, 1991[3]).

	Yield				1000 KW g	Chemical composition d.m. 1989			
	1988*	1989*	1990 I	1990 II	1988	Protein %	Lysine g/16gN	Starch %	Fat %
Reference Yield[a]	(58)	(79)	(59)	(67)	(51)				
Relative Yield (HKg ha^{-1})	100	100	100	100					
Variety									
Bomi	101	102	91	98	52	11.4	3.6	59	2.3
R-1508	89	83	81	84	46	12.9	5.2	54	3.0
Ca429202 (Carla cross)	101	93	96	103	42	10.8	5.6	56	2.9
Ca533601 (Alis cross)	-	102	105	110	43	11.1	5.6	55	3.0

Developing efficient screening tools for simple and complex quality variables

In the IAEA/FAO research programme from 1968 to 1982, there was a considerable effort to develop and test new screening tools for specific chemical variables of nutritional importance [4]. This also included the physical option of Near Infrared Reflection Spectroscopy (NIR) combined with multivariate data analysis (chemometrics) that was introduced for quality control in the cereal industry, including Carlsberg Breweries, in the mid-1970's and was further developed by our group in Spectroscopy and Chemometrics at the Life Science Faculty of the University of Copenhagen from 1991 [5, 6]. In the remainder of this article, we will use data on the mutant *lys3.a* cross breeding material developed at Carlsberg in 1973-1988 to demonstrate how NIR spectroscopy evaluated by Principal Component Analysis (PCA) can visualize changes in seed composition, such as starch content, resulting from genetic improvement. We will also demonstrate how the calcofluor screening method for malt modification and a flotation method for kernel density can be used to test gene expression in a slender endosperm cell wall/low ß-glucan mutant developed at Carlsberg by Sten Aastrup [7].

"Data Breeding" for complex quality traits: Using NIR spectroscopy data and a PCA score plot to select improved segregants

In **Table 2** [6] the chemical composition of 15 genotypes from the Carlsberg *lys3.a* material is presented. The original mutants (Group 4) have lower starch content (48.7%) than normal barley (54.6%). The improved *lys3a* breeding lines (Group 1) and unselected *lys3.a* recombinants (Groups 2 and 3) are intermediate in starch content. A low amide to protein A/P index (**Table 2**) is indicative for high lysine. Comparing NIR spectra in the short wavelength area interval 2260-2380 nm it is seen that the spectral patterns for the normal control Triumph and the original *lys3.a* mutant are quite different, with a plateau for Triumph at 2290 nm (marked **1**) compared to a slope for *lys3.a.* The latter has a characteristic peak at 2347 nm (marked **2**), assigned to fat, that is increased by 55%. The improved semi-commercial *lys3.a* lines, Lysimax and Lysiba, are approaching the normal (Triumph) spectral pattern with regard to the plateau and peak marked **1** and **2**.

The differences in patterns of whole log 1/R multiple scattered corrected NIR spectra (400-2500 nm) of the 15 genotypes are represented as distances in a PCA scoreplot (**Fig. 4B**). It is seen that the improved *lys3a* genotypes with increased starch Lysiba (52.2%) and Lysimax (52.9%) are moved from the position above to the right of the original *lys3.a* mutant (starch 48.5%) towards the position of the normal barley Triumph (starch 58.5%) down to the left. The PCA biplot of chemical data (**Table 2**) in **Fig. 4C** confirms the pattern of the corresponding spectral PCA

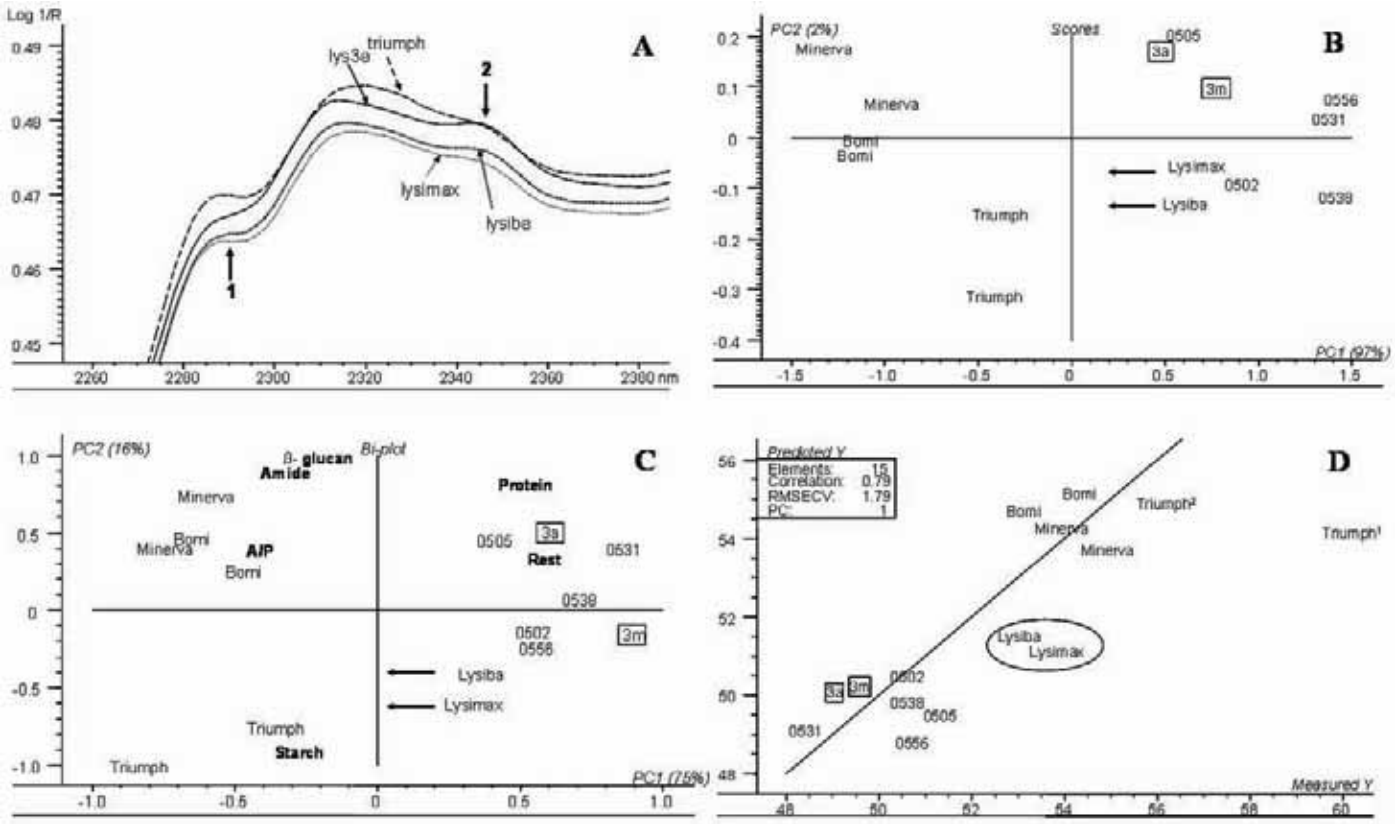

Figure 4 (A) Comparison of the MSC NIR area 2260-2380 nm for samples of Triumph, mutant lys3a and high lysine recombinant lines Lysiba and Lysimax. Numbers are referred to in the text. **(B)** PCA score plot of NIR (MSC) spectra from normal barley (Bomi, Minerva, Triumph), mutants (*lys3.a, lys3.m*) as well as high lysine recombinant lines (0502, 0505, 0531, 0538, 0556, Lysiba, Lysimax). **(C)** PCA biplot of chemical data (protein, ß-glucan, amide, A/P, starch) at the same material. **(D)** PLSR prediction plot of starch (y) by NIR measurements (x).

scoreplot (**Fig. 4B**). NIR spectra indicate patterns of chemical bonds that may be identified from the literature. In the chemical PCA biplot in **Fig. 4C** the variable "starch" is positioned near Triumph, indicating a high level of starch in this cultivar. The move in both PCA´s (**Fig. 4B, 4C**) of the Lysiba and Lysimax genotypes from the position of the original low starch *lys3.a* mutant towards the high-starch variety Triumph indicates a clear improvement in starch content.

The NIR and chemical data sets are combined in the Partial Least Squares Regression (PLSR) starch prediction plot in **Fig. 4D**. This is, in principle, how NIR and NIT spectroscopy are utilized today by plant breeders for chemical prediction, using large calibration sets from the instrument manufacturers to obtain precision when dealing with many varieties and environments. It is possible empirically, and without elaborate calibration by comparison to a high-quality genotype control, to select for *the whole* expression of physical and chemical attributes at the spectral level in a cross-breeding programme by "data breeding" [5, 6].

A complex quality trait such as starch and nutritional value (described here) or malt and baking quality [5, 6] can be represented *as a whole* spectral pattern by NIR and NIT spectroscopy. This can be done without chemical analysis, except for the confirmation and evaluation of the final varieties. It is clear that NIR and NIT spectroscopy combined with PCA and PLSR data analysis (chemometrics) is a revolution in cost-effective breeding for quality in all cereals [5].

Table 2. Average and standard deviation of chemical data for five chemical groups [6]

	Normal (n=6)	Group 1	Group 2	Group 3	Group 4
Protein (P)	11.3±0.4	11.7±0.1	11.7±0.1	12.6±0.2	12.5±0.2
Amide (A)	0.28±0.03	0.21±0.007	0.21±0.007	0.22±0.02	0.23
A/P	15.5±0.9	11.0±0.3	10.9±0.4	10.7±0.8	11.4
Starch (S)	54.6±2.5	52.6±0.5	50.0±0.1	49.4±1.5	48.7±0.2
ß-glucan (BG)	4.7±1.1	3.1±0.1	3.1±0.1	3.1±0.3	2.8±0.5
Rest (100-P+S_BG)	29.5±1.8	32.7±0.5	35.3±0.3	34.9±1.8	36.1±0.5

Group 1=Lysiba, Lysimax; Group 2=502, 556; Group 3=505,531,538; Group 4=lys3a, lys3m

The impact of barley seed mutants on defining functionality in nutrition.

It is often forgotten that the introduction of, for example, a high lysine mutant for practical use in the agricultural and feed industries can be looked upon as a question to nature that could lead to surprising revelations. One such surprise was when we found by NIR spectroscopy [8 and IAEA-CN-167-300 these proceedings] that the three minor lysine mutants Risø 13, 16 and 29 (and three others) contained a ß-glucan content of up to 20% dry matter (d.m.). They had previously been identified as structural mutants for ADP-glucose phosporylation and transport in starch synthesis. Obviously they inherited a surprisingly pleiotropic effect with regard to ß-glucan production that compensated largely for the decrease in starch. Because ß-glucan, as a dietary fiber, does not contribute to metabolic energy, there is no interest in feeding monogastric animals high-ß-glucan/low-starch mutants. However, the high fiber and low caloric content (down to a 50% reduction) in these mutants made them attractive as a source of human food with the potential to reduce cholesterol [8].

Table 3. Feed trial with Piggy high lysine barley [10].

	Protein%	Lysine g/16gN	Fat%	Carb.*	N digest%	Nitrogen retention	Metabolizable energy MJ/kg DM
Lami	13.2	3.64	3.3	62.7	72	22	14.1
Piggy	14.0	5.50	5.5	57.7	69	35	14.0

* Carb. = starch + sugars

The original Risø 1508 *lys3.a* mutant has a reduced ß-glucan content of about 2-4% (compared to 4-6% in normal barley). Nevertheless, Risø 1508 has a 5% absolute decrease in starch and a 2% decrease in digestible energy that should reduce metabolisable energy. The decrease in starch is energy-wise partly counterbalanced by an increase in fat from approx. 2 to 3.5%. Early on in our breeding work with *lys3.a* , we gave a rather pessimistic account on the economic value of the Risø 1508 mutant at the 4th IGBS meeting in Edinburgh in 1981 (Bach Knudsen and Munck [9]). This was because the low energy digestibility of -2 to -4% (in spite of increased fat) that was published in the literature for the Risø 1508 mutant for rats and pigs. Our view was also influenced by the overrating of cereals by EU subventions at the time, favoring available carbohydrates (starch) at the expense of a nutritionally balanced protein. Increasing starch content through the *lys3a* breeding programme at Carlsberg (see Bang Olsen, Stilling and Munck 1991, **Table 1** [3]) and the pig feeding trials that are reported in the following section changed our view.

The mutant lines were instrumental in understanding the functionality of amino acid energy relation ships in feeding trials with pigs using the original Risø 1508 mutant (**Fig. 2**) and with the starch improved *lys3.a* variety Piggy (**Table 3**). Pig trials were undertaken between 1984 and 1989 by H.P. Mortensen and A. Madsen at KVL and by B.O. Eggum at Foulum. The results were summarized in English in 1992 [10]. A pig trial with the Piggy *lys3.a* recombinant and a normal feed barley Lami was made without protein supplementation (**Table 3**). The barley field trial was performed with late nitrogen fertilizer addition to increase protein content from about 11% to 14.0% and 13.2% for Piggy and Lami, respectively. This reduced yield and increased the difference in carbohydrates (starch + sugars) between the two barleys to 5% in absolute terms. Protein (N) digestibility was reduced by 3% and energy digestibility by 2% confirming rat trials [10]. It was surprising, however, that the metabolizable energy of Piggy was only 0.1% less than the Lami control (**Table 3**). The explanation was obtained in a parallel soy bean meal supplementation trial by Mortensen and Madsen [10] employing the same barley samples as in **Table 3**, which was more suitable from a pig production point of view.

Soybean meal could, in restrictive feeding, be reduced from 40.9 kilos per pig down to 25.9 kilos and, in *ad libitum* feeding, from 47.3 to 14.3 kilos with only minor changes in live weight and meat percentage, when supplemented by Piggy barley. The barley consumption was increased by 20.8 and 33.7 kilos respectively. Piggy barley could support adequate growth in pigs from 50 to 90 kilo live weight. It was realized by B.O. Eggum of the Danish Agricultural Research, Foulum, that the soy-bean meal sparing effect of high lysine *lys3.a* Piggy barley could reduce nitrogen pollution in pig lots by 15-20%. This is a considerable amount in Denmark, which produces about 25 million pigs annually. The explanation is that both soybean and barley meal contain large amounts of non- essential amino acids such as glutamine and proline that only can be utilized if adequate amounts of essential amino acids such as lysine, methionine and threonine are available. By introducing the improved Piggy *lys3.a* mutant into pig feeding, the protein concentration in the feed and protein (N) load on the animal could be reduced by 15-20% without compromising meat production. The decreased nitrogen (N) load on the animal spares the energy that is necessary to catabolize the surplus of non-essential amino acids through the liver and the kidneys. The 5% less carbohydrates and 2% less energy digestibility in Piggy measured in fecal trials is thus likely to be compensated by more efficient energy metabolization of the protein. We may conclude that high lysine barley mutant development has contributed to a deeper understanding of animal physiology of practical significance, both nutritionally and environmentally. It is remarkable that the scientific society that focused on protein nutrition in the 1960's identified the same mutant in the 1990's as a gene contributing to reduced nitrogen pollution by pigs.

Using barley mutants to test functionality in malting and brewing

Genetically and chemically defined mutants are not only able to contribute to the quality of raw materials but can also be used to define more precisely the functionality of a quality trait in an industrial process and provide an insight to gene expression in the seed. At Carlsberg in the 1980's, a screening analysis for percent malt modification was developed by staining cell walls (ß-glucan) using the fluorescent dye calcofluor [7]. The malt modification analysis by enzymatic breakdown of barley endosperm cell walls is shown in **Fig. 5A**. Aastrup [7] screened barley for low viscosity (ß-glucan) acid extracts to search for azid mutants. ß-glucan negatively influences wort and beer filtration. A low ß-glucan mutant M-737 from the parent variety Minerva was isolated in which ß-glucan was reduced from 5.9 to 2.7%. There was a resulting reduction in endosperm cell wall thickness by two thirds (**Fig. 5B**). The M-737 mutant increased the speed of malt modification by one to two days because of less modification resistance of the cell walls during enzymatic breakdown during malting (**Fig. 5C**). It was also found that the mutant gene had a pleitropic effect on density (softness) of the seeds (**Fig. 5D**) that could contribute to the increase in malt modification. Because of lower yield the M-737 mutant, it was not exploited for malting. However, it was of fundamental importance to understanding the connection between the raw material and the malting process, as well as to elucidating how important physico-chemical traits were connected by pleiotropy in gene expression. There is a similar story with the proanthocyanidine-free barley mutants developed at Carlsberg by Diter von Wettstein and Barbro Strid, which finally verified the cause of haze in beer [11]. But the latter mutants are now also of commercial importance.

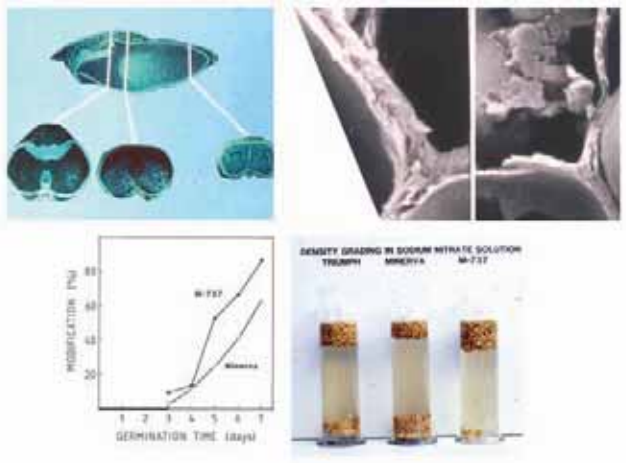

Figure 5 Functionality in barley malting studied by the low ß-glucan, slender cell wall azid mutant Carlsberg M-737 in Minerva reveals faster malt modification and softer, less dense seeds (Aastrup and Munck, 1985). **(A)** Malt modification by break down of cell walls in the barley endosperm visualized by the fluorescent dye calcofluor. **(B)** Scanning electron micrograph of endosperm cell walls. left Minerva (cell wall thickness 1.6 µ; ß-glucan 5.9% d.m.), right M-737 (cell wall thickness 0.5 µ; ß-glucan 2.7% d.m). **(C)** Malt modification% (y) with time (x) for M-737 and the parent variety Minerva analyzed by calcofluor. **(D)** Density grading with flotation for seeds of Triumph, Minerva and M-737.

The future exploitation of cereal endosperm mutants

The classic work on morphological barley mutations at the University of Lund and at the Swedish Seed Association in Svalöf that was started by H. Nilsson Ehle and Å. Gustafson in the 1930's was reviewed by Å. Gustafsson [12] and U. Lundqvist [13] at the 100 Years Jubilee of the Svalöf Institution in 1986. This genetic material is now preserved by the Nordic Genetic Research Center at Alnarp, Sweden, supplemented by the biochemical barley endosperm mutants obtained at Svalöf, Risø and Carlsberg from the 1960's to the 1980's. It is generally recognized that new gene mutations most often give negative pleiotropic effects on yield. It is rare that the mutants can be commercialized directly, as were the erectoides "Pallas" and the early mutant "Mari" introduced by Gustafsson, and by a few of the proanthocyanidine mutants used for malting and brewing discussed by von Wettstein [14]. He also referred to the original valuable *mlo-o* powdery mildew mutation first isolated by Freisleben and Lein in 1942. Only after 45 years of intense recombination breeding could the pleiotropic leaf necrosis problem that lead to deceased yield be solved, and the advantage of the broad and stable resistance be fully exploited. It seems that the majority of scientists are rather pessimistic about the possibility to repair negative pleiotropic effects. They are searching for new genes instead of undertaking the hard work of exploiting the great flexibility of nature to find "a happy home" (gene background) for the mutant by crossbreeding. It seems that only two such successful projects in breeding high lysine varieties with improved yield and seed quality have been fulfilled since 1968: one at CIMMYT for maize [15] and the other at Carlsberg [3, 10] for barley. As was further discussed by us in these proceedings (IAEA-CN-167-300), an open holistic exploratory strategy is necessary in order to evaluate the great complexity of the pleiotropic effects of a mutant gene that can not be forecasted by limited prior scientific knowledge. A coarse overview of the phenotype (Phenome) is now possible by exploiting Near Infrared Spectroscopy [5].

ACKNOWLEDGEMENTS

We are most grateful to IAEA/FAO,Vienna and its staff for supporting endosperm mutant research by many inspiring symposia since 1968 and to Frans van den Berg for assisting us in writing this article and U. Lundqvist in correcting it. Finally, we are deeply indebted to all those who in the past have assisted us in collecting a unique barley mutant collection ideal for research in barley quality.

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Creation and Evaluation of Induced Mutants and Valuable Tools for Pepper Breeding Programmes

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Abstract

Advances in plant molecular biology and screening techniques, integrated with mutation technologies, have allowed for study and better utilization of mutant lines. Application of physical and chemical mutagens in pepper breeding programmes has created mutants with applied value - increased β-carotene levels in fruit, male sterility, lack of anthocyanins, determinate habit, altered fruit shape and changes in fruit position. Recombinant inbred lines (RILs) have been developed and different mutant genes combined in the same genotypes. Mutants demonstrating potential for increased β-carotene levels were selected and exploited as parents for the development of hybrids. Dramatic increases in β-carotene content were found in some of these F_1 hybrids. Results obtained from cytological, biochemical and physiological studies of carotenoid levels, β-carotene hydroxylase activity and chlorophylls, together with observations of the phenotypes of plants and fruits suggested that several mutant lines could be exploited in breeding programmes. Molecular studies allowed us to establish a marker for orange fruit colour useful for MAS.

Introduction

The market justifies the breeding of new cultivars combining high-yield, attractive fruit and high nutritional value. Bulgaria has a leading position in the development of pepper cultivars with improved quality and agronomic characters. Increasing biodiversity by conventional breeding methods has been a difficult and long process. Until recently, pepper improvement programmes were based mainly on utilizing natural sources of germplasm and cross breeding, male sterility and heterosis effects. The breeding of important agricultural characters (yield, resistance to pests and diseases, etc.) have to be performed in parallel with breeding for nutritional value. Our breeding strategy aimed at increasing carotenoid levels, and was also directed towards the selection of other traits, such as productivity, male sterility, plant habit, fruit size and shape, and lack of anthocyanins. Modern techniques for plant characterization, particularly those of molecular biology, were integrated with conventional methods to generate and characterize useful induced mutations [1, 2, 3]. The purpose of this study was the creation and evaluation of valuable pepper mutants through appropriate methods for crop genetic improvement.

Materials and Methods

Investigations were carried out in 2002–2008 at the Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria, with *Capsicum annuum* L. sweet pepper cultivars (cv.) and breeding lines (bl.) for fresh consumption.

Mutants (M) obtained by Tomlekova and colleagues [4] after Ethyl methansulphonate (EMS) and Co^{60} Gamma-ray treatment of wild-type (WT) cv. Hebar were identified by observation of phenotypic alterations. Anthocyanin-free plants were observed at the seedling stage and their phenotype has been further screened.

Physical treatments (120Gy X-rays) on dry seeds of red-fruited (*rf*) local cv. Pazardzhishka kapiya 794 led to obtaining of a mutant (of, M^{of}), with orange-coloured mature fruit [5], and a cv. Oranzheva kapiya was developed by Daskalov and colleagues [6].

Mutants lacking anthocyanins (*al*) were obtained following gamma irradiation of cv. Zlaten medal 7. Mal plants with early and high yield, more attractive fruit and better flavor, were developed in cv. Albena by Daskalov [7]. After the incorporation of the mutant character orange fruit color (*of*) into genotypes bearing the al mutation a series of recombinant inbred lines (RILs; genotypes 32, 33, 34, 35, 36) were developed ($M^{of,al}$) [5, 9]. Near isogenic lines (NILscolournge) were advanced to M12-15 and used for crosses with the corresponding WT, to produce WTxM and MxM F_1 hybrids. Mutant genes introduced into elite pepper M^{of} material were studied for their influence on β-carotene content in mature fruit.

Male sterile mutant Zlaten medal *ms8* (28) (M^{ms8}) had been obtained by the application of 120Gy Rö-rays to the fertile (f) local cv. Zlaten medal 7 (WT^{f}) [8, 10, 15]. The bl. 28^{ms8} and $30^{Ms,of,al}$ were crossed by us to combine *of, al*, and *ms8*. The type of segregation (fertile (f): sterile (s) plants) was defined in 124 F_2 plants. These plants were obtained from seeds produced from different isolated fruits and plants of the same cross. Pollen fertility or lack of pollen in anthers in the blossoming period was assayed in 4% acetocarmine and glycerol (1:1) and microsporogenesis by squashing anthers in 4% acetocarmine.

Fruits from 20 individual plants of each genotype listed in **Table 1** at the same stage of botanical maturity were used for biochemical analyses of β-carotene content using column chromatographic absorption [11]. A three-year biochemical selection of bl. toward stable β-carotene levels was conducted.

Photosynthetic pigments, chlorophylls (*Chl*), and total carotenoids in fruit were also analyzed at botanical maturity, by the method of Wettstein [12]. Analysis of the inheritance of β-carotene content in fruit was done according to Genchev, *et al.* [13].

The β-carotene hydroxylase (*CrtZ*) activity was analyzed in crude extracts of green and mature lyophilized fruit of one WT and two M lines, especially searching for differences in β-carotene levels by detection of the conversion of β-carotene (orange pigment) to β-cryptoxanthin (yellow xanthophylls). Molecular characterization of the genes encoding two hydroxylases *CrtZ* and *CrtZ-2* was conducted. Genomic DNA from M^{of} and WT^{rf} was isolated using CTAB protocol, and used for gene-specific PCR according to FAO/IAEA protocols [14].

Results and Discussion

The population raised from M_1 seeds from WT cv. Hebar treated with EMS and Co^{60} irradiation presented several morphological changes in the M_4 generations (**Fig. 1**).

Phenotypes were screened in M_2 plants and mutant characters selected

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **187-190**

as valuable were fixed in further generations. A mutant with determinate habit was selected and maintained.

Selected M plants included genotypes lacking anthocyanins, those with erected fruit, erected fruit in a bunch and altered fruit shape. Confirmation of these traits was performed until the M_4 generations for further development of advanced mutant lines.

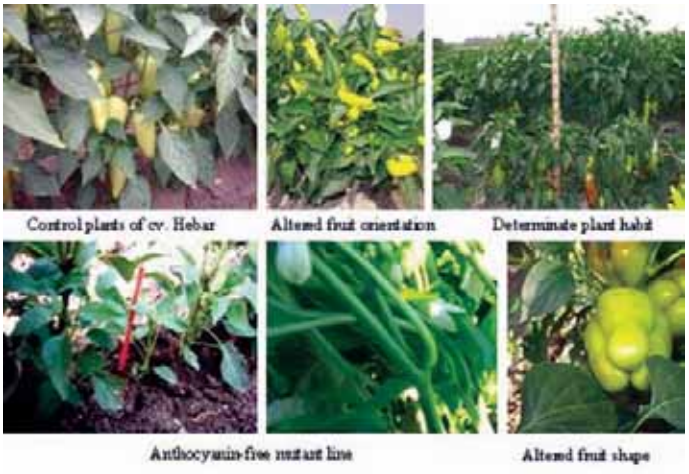

Figure 1 Induced pepper mutants in the M_4 generations following EMS treatment and Co^{60} irradiation.

The cytological results (**Fig. 2 a, b, c**) undertaken on the F_2 segregating progeny (28x30) showed that the average ratio of fertile: sterile plants was 4.24:1, which is close to the expected 3:1 ratio. The expression of the mutant gene *ms8* was stable and no pollen formation in the anthers of the studied sterile plants in both - the mother line 28 and the sterile F_2 plants was observed, which is consistent with previous investigations [8]. The anthers were deformed and without pollen grains. Results of the meiotic analysis in pollen mother cells of sterile plants from line 28 revealed that lethality of the cells occurred after second telophase, by blocked cytokinesis and tetrad formation in the ms_8ms_8 plants was not observed.

Figure 2 Anthers of F_2 plants: fertile (a) with pollen grains (b) and sterile (c).

The biochemical data obtained in 2005 of the orange-fruited mutants demonstrated that the β-carotene content was genotype-dependant (**Table 1**). Comparative analyses between the groups of M^{of} and WT^{rf} revealed much higher accumulation of β-carotene in the orange-fruited (*of*) lines. All M^{of} had higher β-carotene content (12.57 to 19.58 mg/100 g fresh matter (*fm*)) than the WT^{rf} (bl. 28, 31), which had red fruit (*rf*; 6.68 and 4.30 mg/100 g fresh matter (*fm*)). The β-carotene levels in different F_1 hybrids ($M^{of}xWT^{rf}$; $M^{of}xM^{of}$) were also greater than the WT^{rf}. The highest concentrations were obtained when two parental M^{of} were hybridized. The β-carotene content was the greatest in the F_1 [35x17] hybrid (41.05 mg/100 g fm). The very high β-carotene levels of the F_1 hybrids were preserved in three consecutive years, despite of the variation in this trait. Higher β-carotene levels in most of F_1 hybrids were established combined with lower variation in this trait compared to the corresponding parents.

Genotypes that participated in the studied crosses were selected for exploitation as parents for the development of hybrids. Line 35, followed by lines 31 and 30 were recommended. The F_1 hybrids obtained with bl. 31 could contribute to improved market performance due to the preference of red-fruited peppers. Variability in the β-carotene content of M^{of} with the same origin indicated that besides this mutation, there are other genetically-determined factors contributing to increase in this compound, reported for other pepper genotypes [19]. The presence of mutant gene in M^{of}, the observed pyramiding of responsible genes and their interaction in some of the F_1 hybrids demonstrated a greater potential for increased β-carotene levels. Despite the statistical significant difference (95%) between WT^{rf} and M^{of} groups, the inheritance of the β-carotene trait varied from partial dominance to over dominance in the same F_1 hybrids. In each WT^{rf} and M^{of} genotype a further selection towards the β-carotene levels was conducted to decrease the variation of the target (data not shown).

Table 1. Variation in β-carotene content per 100g fresh fruit in individual plants of pepper genotypes

Genotype-Parents and F1 hybrids			β-carotene mg/100 g fm		Error of the difference	
Line number		Fruit color	x	±S x	$t_{experimental}$	VC%
P_1	28	red	6.68***	0.71	4.53 (F_1&P_1)	35.32
P_2	29	orange	12.57***	1.01	4.75 (P_2&P_1)	26.74
F_1	[28x29]	red	12.20	0.98	-0.26 (F_1&P_2)	25.60
P_1	28	red	6.68***	0.71	4.65 (F_1&P_1)	35.32
P_2	30	orange	15.84***	1.55	5.36 (P_2&P_1)	31.00
F_1	[28x30]	red	17.01	2.10	0.44 (F_1&P_2)	27.64
P_1	32	orange	12.80***	1.38	6.77 (F_1&P_1)	35.98
P_2	31	red	4.30***	0.45	-5.81 (P_2&P_1)	28.05
F_1	[32x31]	red	23.98***	0.89	19.62 (F_1&P_2)	10.54
P_1	35	orange	13.62**	1.07	3.26 (F_1&P_1)	24.92
P_2	31	red	4.30***	0.45	-7.98 (P_2&P_1)	28.05
F_1	[35x31]	red	27.87***	4.23	5.53 (F_1&P_2)	26.32
P_1	32	orange	12.80*	1.38	2.68 (F_1&P_1)	35.98
P_2	17	orange	19.58*	2.00	2.78 (P_2&P_1)	32.33
F_1	[32x17]	orange	18.89	1.79	-0.25 (F_1&P_2)	25.13
P_1	35	orange	13.62***	1.07	11.36 (F_1&P_1)	24.92
P_2	17	orange	19.58*	2.00	2.62 (P_2&P_1)	32.33
F_1	[35x17]	orange	41.05***	2.16	7.28 (F_1&P_2)	10.54

Df=18, $t_{critical}$, $P_{5\%}$ (2.1), $P_{1\%}$ (2.88), $P_{0.1\%}$ (3.92); VC=variation coefficient%; x=Average value; ±S =Error of x

The quantity of chlorophylls, and total carotenoid content, was genotype-dependent. Effects of mutation in chlorophylls and total carotenoids in fruit were detected. *Chl a* contributed most to the increase of the total chlorophylls. Despite the increase in β-carotene content, the rest of carotenoids contributed to a decrease in the total carotenoid content (data not shown).

On the basis of the data for β-carotene and total carotenoid levels, a hypothesis for a mutation that affected a gene encoding an enzyme responsible for hydroxylation of β-carotene to β-cryptoxanthin was erected. Significantly less hydroxylase (e.g. *CrtZ*) enzyme activity in ripe fruit was noted in the M^{of} compared to the control WT^{rf} (**Fig. 3**). This enzyme is responsible for the hydroxylation of β-rings and the conversion of carotenes into xanthophylls. Thus, data from analysis of enzyme activity support the hypothesis.

Molecular studies have also supported this hypothesis. PCR reactions with different gene-specific primers were undertaken for two hydroxy-

lase structural genes known to be involved in pepper biosynthetic pathway (**Table 2**).

Both hydroxylases are expressed in pepper [16], in tomato [17], and localized in the green tissue and flower (Ronen, *et al.*, 2000, quoted by [18]). A band from *CrtZ* was amplified using primer combinations *CrtZ*-C, D, D/C and E in all WT^{rf} but was absent in M^{of} (**Fig. 4**). Fragments amplified from *CrtZ*-2 in all WT^{rf} and M^{of} showed monomorphic DNA profiles. This result was confirmed by a following sequencing of fragments isolated from *CrtZ* and *CrtZ*-2 after amplification with the *CrtZ*-C/C.

Two DNA fragments were amplified and isolated from each WT^{rf} corresponding to *CrtZ*-2 and *CrtZ* genes and a unique fragment - from M^{of} corresponding to *Crt-2*. A mutation occurring in the 3'-terminal region in the mutant was very probable. The noted primer combinations could be efficient as gene-marker for selection performing towards orange fruit color in M^{of}.

Table 2. PCR amplification with specific primers for the gene *CrtZ* and *CrtZ*-2.

Gene amplified	Primer design by cDNAbp	Primer sequences	Results from fragments
CrtZ-C/C	607-885	F GAG CTG AAC GAT ATT TTT GCC	Band ~620bp from *CrtZ*-C/C in WT; absent in M;
CrtZ2-C/C	586-864	R TAG GAA CAA GCC ATA TGG GA	~760bp from *CrtZ-2*-C/C present in WT and in M
CrtZ-D/C	526-885	F AGA TGG GCG CAT AGA GCA CTA	Band from *CrtZ*-D/C ~700bp in WT;
		R TAG GAA CAA GCC ATA TGG GA	absent in M
CrtZ-D/D	526-866	F AGA TGG GCG CAT AGA GCA CTA	Band from *CrtZ*-D/D ~750bp in WT;
		R ACC CCA TCA AAT TTG TCC GA	absent in M
CrtZ-E/E	20-866	F CGT ACA TGG CTG CTG AAA TT	Band from *CrtZ*-E/E ~1400 in WT;
		R ACC CCA TCA AAT TTG TCC GA	absent in M
CrtZ-C/D	607-866	F GAG CTG AAC GAT ATT TTT GCC	Band from *CrtZ*- C/D ~580 in WT;

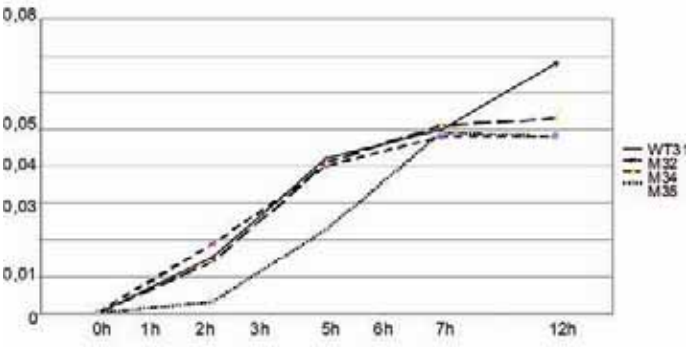

Table 2. PCR amplification with specific primers for the gene *CrtZ* and *CrtZ*-2.

	R ACC CCA TCA AAT TTG TCC GA	absent in M

* monomorphism - (-); polymorphism - (+)

Figure 3 *CrtZ* activity registered in pepper fruit of WT^{rf} and M^{of} at botanical maturity. x - time of registration of the enzyme activity – 2, 5, 7, 12, 24 hours after starting of the reaction; y - decrease of absorption values.

Conclusions

Application of EMS and Co^{60} created mutations of applied value, such as determinate habit, lack of anthocyanins, and changes of fruit shape and position. Recombinant inbred lines were developed and different mutant genes were combined in the same genotypes.

Cytological studies demonstrated the stability of the expression of the gene *ms8* determining male sterility.

Fruit from pepper mutants and wild-type plants differed in their carotenoid content. In the fruit of the mutant lines, β-carotene levels increased dramatically. Conversely, a decrease in the accumulation of total carotenoids, due to a decrease in xanthophyll formation, was found in the mutants. A reduction in *CrtZ* enzyme activity, which converts the β-carotene to β-cryptoxanthin, was observed in the mutants. Molecular investigations suggested changes occurring in the 3'–terminal region of the *CrtZ* gene could be responsible for reducing the hydroxylase enzyme activity of the mutants.

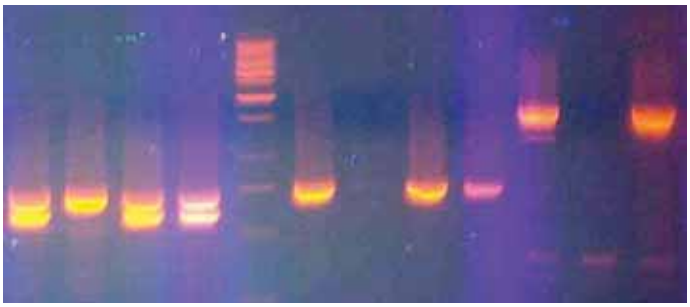

Figure 4 PCR fragments in sweet pepper WT^{rf} and M^{of} with gene specific primer combinations: 1-4–*CrtZ* and *CrtZ-2* C/C (WT_1, M, WT_2, WT_3); 5-DNA Ladder (250, 500, 750, 1000 bp); 6-9-*CrtZ* D/D (WT_1, M, WT_2, WT_3); 10-12-*CrtZ* E/E (WT_1, M, WT_2).

This study allowed breeding strategies to exploit different mutant lines for developing F_1 hybrids with increased β-carotene content. The C and D/C combinations could be used as a marker for high β-carotene for performing marker-assisted selection. The presence of a fragment from *CrtZ-2* amplified in the mutant genotypes by primer combination C is very useful as an internal standard (i.e. multiplex) co-dominant allele-specific marker (null).

ACKNOWLEDGEMENTS

Work was performed within the CRP D23024 on Physical Mapping Technologies for the Identification and Characterization of Mutated Genes Contributing to Crop Quality Research Contract N°12227 with FAO/IAEA.

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Induced Mutagenesis for Oil Quality Enhancement in Peanut (*Arachis hypogaea* L.)

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Abstract

Increasing the ratio of oleic to linoleic acid (O/L) in peanut (*Arachis hypogaea* L.) significantly improves the nutritional and quality attributes of the crop. The lack of sufficient genetic variation in fatty acid composition, particularly the O/L ratio, in peanut germplasm and presently grown cultivars make the creation of such variability necessary. Mutation breeding of peanut was initiated with the objective of identifying stable peanut mutants with altered fatty acid composition for improved oxidative stability and nutritional quality. Seeds of peanut cultivars 'GPBD-4' and 'TPG-41' were treated with γ-radiation and/or ethyl methane sulphonate (EMS). Randomly selected mutants were advanced based on single plant selection up to the M_4 generation and the harvest of M4 plants was evaluated for fatty acid composition by gas chromatography. Highly significant variation for palmitic, stearic, oleic, linoleic and arachidic acid was observed. EMS (0.5%) and 200Gy treatments were found to be effective in increasing the variability in fatty acid content in GPBD-4 and TPG-41, respectively. The variability was skewed towards high levels of oleic acid (38-67%) and low levels of linoleic acid (15-41%). Mutants selected for improved oil quality were significantly superior for O/L ratio and had reduced palmitic acid. Oil with reduced palmitic acid and increased O/L ratio is desired nutritionally. Hence, these mutants can be exploited for the improvement of oil quality. The mutants GE-87 and T3-105 recorded the highest O/L ratios, of 4.30 and 3.91, compared to control values of 1.75 and 2.60, respectively. A significant negative correlation between oleic acid and linoleic acid, palmitic acid and iodine values, and weak inverse relationship with oil content indicates the possibility of selection for improved fatty acid composition. These high oleic acid lines could be utilized further in breeding programmes for improvement of peanut oil quality.

Introduction

The peanut (*Arachis hypogaea* L.) is cultivated in most tropical and subtropical regions. Because of extensive production for cooking oil, the quantity and quality of the oil are extremely important considerations in cultivar development. Peanut genotypes contain approximately 50% oil [1], which becomes rancid upon exposure to air and heat due to oxidation [2, 3]. Consequently the shelf life of oil and products that contain peanut and peanut oil, is limited. The oxidative stability and shelf-life of peanut oil is influenced by the concentrations of specific fatty acids [4, 5]. In general, saturated fatty acids are less susceptible to oxidation than less saturated fatty acids. Two fatty acids, oleic and linoleic, comprise over 80% of the oil content of peanut. Of these, linoleic acid is less saturated and less stable than oleic acid. There is a strong negative correlation between linoleic acid content and oil stability in peanut [6, 7]. In addition to increasing the stability of peanut oil, increasing the O/L ratio appears to have health benefits as well. Research has associated high oleic acid with lowered blood serum cholesterol, especially low density lipoproteins (LDL) in humans [8] and a reduction in recurrent myocardial effects when oleic acid levels are increased in plasma fatty acids [9].

Increasing the O/L ratio in peanut seems to have positive effects on peanut quality and nutritional value. The majority of peanut cultivars average 55% oleic acid and 25% linoleic acid [10]. The available germplasm and breeding lines screened to date have indicated very limited genetic variability for O/L ratio (0.8-2.50). No genotypes were found in either National (NRCG, Junagadh, India) or International (ICRISAT, Hyderabad, India) germplasm collections of peanut with high O/L ratios comparable to the natural mutants isolated in Florida, USA [11]. These mutants as well as their derivatives are highly protected and patented. Thus they are not available for use in international breeding programmes. Therefore, it is essential to create new genetic variability for this value-added trait for the improvement of peanut oil quality. The occurrence of natural mutations, possibility of induced mutations and significant achievements made in different oilseed crops suggest that if concerted efforts are made with the specific objective of increasing oleic acid through induced mutations, it is achievable.

The present study was initiated with the objective of developing peanut lines with a beneficially altered fatty acid composition in two indigenous peanut varieties using induced mutagenesis.

Materials and Methods

Two Spanish Bunch genotypes viz., GPBD-4 and TPG-41 were used for mutagenic treatments.

Mutagen treatments

Seeds of peanut cultivars GPBD-4 and TPG-41 were treated with γ-radiation and ethyl methane sulphonate (EMS). Uniform size seeds of each cultivar were used for treatment. Treatments (500 seeds per treatment) consisted of two different doses of γ-radiation (200 and 300Gy) and EMS (0.5%). Untreated seed of the respective cultivars were used as a control. Seeds were irradiated with γ-radiation at Bhabha Atomic Research Center (BARC) Mumbai, India. EMS solution was prepared in 0.1 M phosphate buffer (pH = 7.0). Seeds were presoaked in distilled water for eight hours to allow uptake of EMS. Presoaked seeds were then treated with EMS for two hours at room temperature in cloth bags. Treated seeds were rinsed in running tap water for four hours and sown in field plots along with untreated controls. The seeds were sown in a randomized complete block design, in five replications, with spacing of 30cm between the rows and between plants. Recommended agricultural practice was followed. The M_1 plants were harvested on a single plant basis. In the M_2 generation one hundred progenies of each treatment in both genotypes were selected randomly and advanced to the M_4 generation on single plant basis. The harvest of M_4 plants was used for fatty acid analysis.

Fatty acid analysis

In the M_4 generation a hundred mutant progenies per treatment were analyzed for fatty acid composition, following the extraction and esteri-

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **191-194**

fication method [12]. The oil content of selected mutants with high O/L ratios was determined by nuclear magnetic resonance techniques [13].

Gas chromatography analysis

A gas chromatograph, model GC-2010 equipped with automatic sample injector AOC-20i, flame ionization detector (Shimadzu, Kyoto, Japan), fitted with a narrow bore capillary column Rtx-wax (film thickness-0.25µm; I. D.-0.25 mm ; length-30 m) was used to separate methyl esters. The initial column temperature was set at 170°C and held for three minutes, then programmed to increase by 10°C per minute to a final temperature of 230°C, at which it was held for one minute. Injector and detector temperatures were both set at 250°C. The flow rates for nitrogen (carrier gas), hydrogen and air were 45, 40 and 400 ml per minute, respectively. The fatty acid methyl esters were identified by comparison with the retention times of a standard methyl ester fatty acid mixture (Sigma, Aldrich). The concentration of each fatty acid was determined by normalization of peak areas and reported as per cent of the total fatty acids.

Statistical analysis

Mean, range and variance were calculated in the M_4 generation for each mutagenic treatment and control to determine the extent of variability created. Two-way analysis of variance was computed to ascertain fatty acid differences in the M_4 generation of all treatments and control using SPSS (version 10). Significant differences between and within treatments means were determined using least significant difference (LSD) values. The oil quality parameters viz., O/L ratio, unsaturated to saturated ratio (U/S) and iodine value (IV) were computed as follows:

1. O/L ratio =% oleic (C18:1) /% linoleic (C18:2)
2. U/S ratio =% (oleic + linoleic + ecosenoic) /% (palmitic +srearic + arachidic + behenic + lignoceric).
3. Iodine value = (% oleic x 0.8601) + (% linoleic x 1.7321) + (% ecosenoic x 0.7854) [14]

In high oleic acid mutants, simple correlation coefficients were also calculated for fatty acid content and different quality parameters using SPSS (version 10).

Results and Discussion

Variability in fatty acid composition

The utility of any vegetable oil is largely determined by its fatty acid composition. Therefore, genetic variability in fatty acid composition is pre-requisite for any breeding programme aimed towards oil quality improvement. As a result of mutagenic treatments, a wide range of variability for all the fatty acids was observed in M_4 seeds. Oleic acid and linoleic acid represented ca. 90% of the fatty acid profiles of peanut and exhibited more variability compared to other fatty acids (**Table 1**). Mean values of treatments particularly in GPBD-4 were higher than corresponding control mean values for most of the traits under study. Interestingly, the range and variance for oleic and linoleic acid was many fold higher than to the parents (control), indicating substantial induced genetic variability for these traits. EMS (0.5%) induced greatest variability for important oil quality traits in GPBD-4, followed by the 300Gy and 200Gy irradiation treatments. By contrast, in TPG-41, doses of 200Gy and 300Gy induced greatest variability for important oil quality traits, followed by EMS (0.5%). These results indicated differential responses of the genotypes to mutagen treatment and the potential of mutagens to create genetic variability for oil quality traits in peanut. The cultivar TPG-41 was found to be more sensitive to mutagenic treatments than GPBD-4, as higher variance values were recorded. Thus it is suggested that selection of a mutagen-sensitive genotype and appropriate mutagens could create maximum genetic variability. The highest mean and range for O/L ratio was observed in TPG-41 (2.40) and GPBD-4 (1.0-4.3) treated with EMS (0.5%), respectively. The iodine value, which is inversely proportional to oil stability, was found to be low in mutagenized populations compared to controls, and the lowest mean iodine value was observed in TPG-41 treated with 200Gy (90.70). Treatment

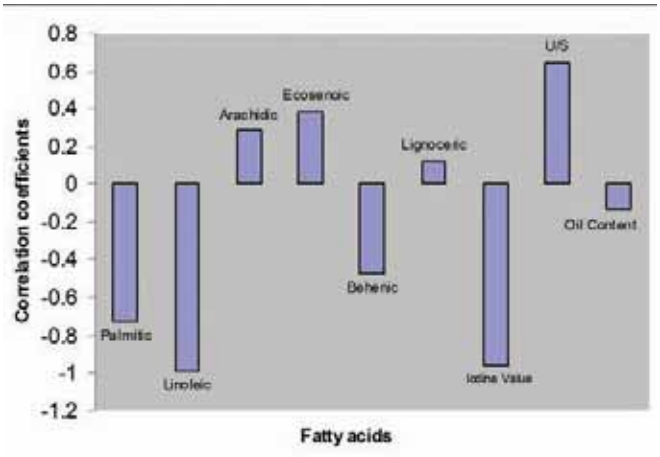

Figure 1 Correlation between oleic acid and other fatty acids in peanut.

Table 1. Fatty acid variation in M_4 generation in peanut induced by mutatagenesis

Genotypes	Fatty acid	Treatments	Mean	Range	Variance
GPBD-4	**Oleic acid (O)**	Control	50.57	49.0-52.0	0.8
		EMS (0.5%)	54.73	41.0-66.6	26.91
		200 Gy	55.66	48.0-65.0	9.46
		300 Gy	52.19	44.0-65.0	13.52
TPG-41		Control	58.00	57.0-58.0	0.35
		EMS (0.5%)	56.40	48.0-65.0	16.25
		200 Gy	58.61	38.0-64.0	42.10
		300 Gy	59.08	39.0-65.0	32.42
GPBD-4	**Linoleic acid (L)**	Control	29.09	28.0-30.0	0.72
		EMS (0.5%)	26.93	15.0-36.0	20.23
		200 Gy	28.96	16.0-31.0	7.64
		300 Gy	28.20	16.0-34.0	9.97
TPG-41		Control	22.51	21.0-23.0	0.75
		EMS (0.5%)	24.40	16.0-31.0	12.07
		200 Gy	26.45	18.0-41.0	32.30
		300 Gy	26.21	17.0-41.0	23.88
GPBD-4	**O/L**	Control	1.74	1.0-2.0	0.01
		EMS (0.5%)	2.03	1.0-4.3	0.35
		200 Gy	1.86	1.5-4.0	0.19
		300 Gy	1.56	1.0-4.0	0.25
TPG-41		Control	2.58	2.0-2.7	0.02
		EMS (0.5%)	2.40	1.0-4.0	0.30
		200 Gy	2.18	1.0-3.5	0.35
		300 Gy	2.70	1.0-3.8	0.29
GPBD-4	**Iodine value**	Control	94.63	93.0-96.0	1.06
		EMS (0.5%)	91.21	82.0-101	12.90
		200 Gy	93.36	84.0-95.0	5.39
		300 Gy	94.63	83.0-100	1.06
TPG-41		Control	91.00	88.0-90.0	1.04
		EMS (0.5%)	91.60	85.0-97.0	7.65
		200 Gy	90.70	88.0-95.0	19.66
		300 Gy	93.66	87.0-103	13.12

Table 2. Fatty acid profile and oil content of mutants with the greatest O/L ratio selected in the M_4 generation

Mutants	Fatty acid profile (%)											Oil content (%)
	16:0	18:0	18:1	18:2	20:0	20:1	22:0	24:0	18:1/ 18:2	IV[a]	U/S[a]	
GE-87[b]	8.25	0.97	66.58	15.53	1.50	1.58	3.70	1.87	4.30	85.41	5.14	47.35
GE-112	7.32	2.78	64.94	15.78	3.12	0.70	3.86	1.45	4.12	83.74	4.40	47.10
GE-53	9.38	2.38	63.40	17.32	1.46	1.30	3.10	1.61	3.66	85.55	4.57	46.86
G2-229	7.76	1.77	65.32	16.53	1.24	1.60	4.01	1.74	3.95	86.07	5.05	47.26
G2-123	7.30	3.75	63.81	16.66	1.85	1.06	4.07	1.48	3.83	84.57	4.42	47.08
G3-18	7.98	2.70	63.96	16.27	1.70	1.50	3.95	1.92	3.93	84.37	4.48	48.20
G3-75	8.32	2.21	65.02	16.08	1.38	1.47	3.71	1.77	4.04	84.93	4.75	46.75
G3-280	7.85	3.07	62.08	18.60	1.73	1.21	4.06	1.42	3.34	86.56	4.52	46.24
TE-231	8.62	2.04	64.22	17.46	1.34	1.22	3.15	1.52	3.68	86.44	4.97	47.00
T3-105	7.50	4.26	65.24	16.70	1.76	0.80	2.62	1.10	3.91	85.67	4.80	46.25
GPBD-4	10.12	1.80	50.67	29.00	1.65	1.30	3.64	1.71	1.75	94.83	4.28	47.70
TPG-41	10.85	1.77	58.67	22.61	1.34	1.21	3.10	1.73	2.60	90.58	4.40	45.25
$LSD_{0.05}$	1.02	0.95	2.36	1.84	0.65	0.48	0.51	0.44	0.98	2.16	0.88	1.13

[a] IV-iodine value, U/S-ratio of unsaturated to saturated fatty acids
[b] First letter and second letter/digit indicates the genotype and mutagen treatment, respectively (GE-87: GPBD-4, EMS treatment, progeny 87)

with EMS in higher concentrations, as well as combined treatment of both γ-radiation and EMS, increased the variability for fatty acids particularly oleic and linoleic acid content in soybean oil [15].

In the M_4 generation, out of 55 distinct mutants identified for altered fatty acid composition, the 10 mutants with greatest O/L ratio are presented in **Table 2**. The most distinguishable feature of the fatty acid profiles of these mutants was the relative contribution of oleic and linoleic acids to the total. The 10 mutants were significantly superior in O/L ratio, which ranged from 3.34 - 4.27 compared to 1.75 (GPBD-4) and 2.60 (TPG-41). The mutant GE-87 recorded the highest O/L ratio of 4.30, highest U/S ratio of 5.14, and lowest iodine value of 85.41, with an oil content of 47.35% comparable to the control (47.70%). The best mutant in the TPG-41 mutagenized population was T3-105 recording an O/L ratio of 3.91, a U/S value of 4.8, an iodine value of 85.67, and comparable oil content to its parent. The rest of the mutants had O/L ratios of 3.44 to 4.12. Palmitic acid, the principal saturated fatty acid in plant oils, is known to be associated with increased levels of blood cholesterol, arteriosclerosis and high risk of coronary heart disease [16]. Interestingly, the mutants identified here had significantly reduced palmitic acid. The mutant GE-112 recorded the lowest palmitic acid content of 7.32%, compared to a parental value of 10.12%. These results clearly indicate that the mutants are significantly superior for oil quality and nutrition. Mutation breeding has been extensively applied to modify the fatty acid composition of soybean [17] and canola [18].

Correlation among fatty acids and oil content

The correlation coefficient between oleic acid and other fatty acids and total oil content are presented in **Fig. 1**. The highest correlations were noted for the percentage oleic and linoleic acids (r = -0.99) and for percentage oleic and palmitic acids (r = -0.73). A positive relationship was observed between the percentage oleic and eicosenoic (r =0.38), arachidic (r = 0.28) and lignoceric (r = 0.12) acids and significant inverse relationships between percentage oleic and behenic acids (r = -0.48). Oleic acid content was inversely related to iodine value (r = -0.96) and positively correlated to the ratio of unsaturated to saturated fatty acids (r = 0.64). The percentage of oleic acid showed a weak inverse association with oil content (r = -0.13). The strong negative correlation between the percentage of oleic and linoleic acid results from them being the main acyl groups in the oil, so that one cannot increase much without a decrease in the other. The negative correlations between the percentages of oleic and linoleic and palmitic acids [19, 20] and no correlation, or weak inverse relationship between percentage oleic acid and oil content have also reported by other researchers [21]. These findings suggest that it is possible to obtain varieties with very high oleic and very low linoleic and palmitic acids with no significant loss in oil content of seed.

The mutant lines identified in this study were found to be promising from the viewpoint of oil quality and stability, and had an oil content comparable to the control. Now, they need to be tested for their productivity and adaptability. In currently grown cultivars, the O/L ratio ranges from 0.8 to 2.5 [22] and the accessions and germplasm screened in peanut have indicated low variability for fatty acid profile. In this regard, the mutants identified in the present study can be utilized as a potential genetic resource for improving peanut oil quality.

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Protein Content in High-Protein Soybean Mutants in Thailand

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Abstract

Two studies have been initiated to enhance nutritional quality of seed protein content in soybean varieties using induced mutation techniques. Approximately 5,000 seeds of uniform size of each variety were irradiated with Gamma-rays at a dose of 200Gy at Kasetsart University. The Kjeldahl method was used to analyze seed protein percentages. **Experiment I.** Seed of three soybean varieties, Chiang Mai 60, SSRSN35-19-4 and EHP275 were irradiated. M_1 to M_4 generations were grown at Nakhon Ratchasima Field Crops Research Center during 2004–2007. The Pedigree method of selection was used. In the M_2, M_3 and M_4 generation, selected mutant lines had 1.9–2.6%, 1.5–2.3% and 0.8–2.2% higher seed protein content than the three checks, respectively. In a preliminary trial, the high protein mutant lines were tested for their protein yield. The mutants had average protein content of 42.5%, 42.4% and 42.9% whereas the check varieties had average protein content of 41.8, 40.3% and 41.9%, respectively. There were six, 18 and eight promising mutant lines selected from Chiang Mai 60, SSRSN35-19-4 and EHP 275, respectively. The mutant lines produced both high seed protein content and high yield. They will be tested in replicated trials in the research centers and farmer fields. **Experiment II.** cm9238-54-1 (ST) was a promising soybean line to be released for farmers. It gave 5–10% higher grain yield than cv Chiang Mai 60, the most popular variety in the northern and central regions. However, this line was susceptible to Soybean Crinkle Leaf (SCL) Disease. M_1 plants generated by induced mutation were grown in the dry season of 2003 at Sukhothai Technical and Production Resources Service Center (TPRSC). The M_2 and M_3 seed were sown in the dry and rainy seasons of 2004 and selected M_4 lines were tested for grain yield in four environments, dry and rainy season 2005, rainy season 2006 and dry season 2007 at Lop Buri TPRSC. From the 2006 to 2007 trials, six selected lines were found to be resistant to SCL in laboratory tests and gave 74–81% higher grain yield than that of the original parent. In addition, they had 2.1–4.0% and 2.1–7.5% higher seed protein content than a check variety, Chiang Mai 60, respectively and had 0.5–2.0% and -1.0–3.3% higher seed protein content than another check variety, SJ4, respectively. The mutants had average protein content of 38.5–43.8%, while the two check varieties had average protein content of 36.3–39.9%.

Introduction

Seeds of soybean (*Glycine max* L.) are important to the agriculture economy because of their high quality and nutritional protein. In Thailand, soybean has been cultivated following the rainy season rice harvest, particularly in the northern region for centuries. However, annual soybean production over the last five years has been able to supply only 15–20% of the country's demand. A total number of 16 varieties have been officially released in Thailand since 1965, with the most popular varieties being Chiang Mai 60 and SJ4. These two varieties had average grain protein contents lower than 40%. An increase in seed yield and quality of soybean was achieved by pre-planting gamma irradiation [2]. Soybean variety improvement for increased nutritional quality protein content using induced mutation was initiated. It is possible to select mutant lines with seed protein content higher than their respective parents by at least 1–2%. The objective of the two studies presented here, which were undertaken in the IAEA/RAS/5/040 project, was to enhance protein content in soybean lines using induced mutation techniques.

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Experiment I

Materials and Methods

Approximately 5,000 seeds of uniform size of each three soybean varieties, Chiang Mai 60, SSRSN35-19-4 and EHP275, were irradiated with Gamma-rays at a dose of 200Gy, as recommended by IAEA [3], at Kasetsart University. M_1 to M_4 generations were grown in the field at Nakhon Ratchasima Field Crops Research Center (FCRC) from 2004 to 2007. The Pedigree method of selection was used. The high protein mutant lines were tested for their protein yield in replicated trails. The Kjeldahl method [1] was used to determine seed protein percentages. Measurement of total nitrogen by Kjeldahl analysis is the historical reference method for determination of the protein content of dairy products. For quality assurance of the data, duplicated samples, laboratory-fortified matrix samples, and an internal reference, soybean seed sample were also analyzed and included in each batch of samples. The protein content was calculated as the total nitrogen (N) content multiplied by 6.25.

Table 1. The seed protein content in the M_2, M_3 and M_4 generations and in a preliminary trial at Nakhon Ratchasima Field Crops Research Center from 2004 to 2007.

Varieties	Seed protein (%)			
	M_2	M_3	M_4	Preliminary trial
Mutant CM 60	45.16	43.87	42.77	42.52
Original CM 60	43.01	42.36	41.99	41.75
Difference CM 60	+2.15	+1.51	+0.78	+ 0.77
Mutant SSRSN 35-19-4	44.90	45.19	44.39	42.36
Original SSRSN 35-19-4	42.30	42.85	41.71	40.32
Difference SSRSN35-19-4	+2.60	+2.34	+2.02	+2.04
Mutant EHP 275	44.79	43.33	43.14	42.94
Original EHP 275	42.89	41.49	41.25	41.93
Difference EHP 275	+1.90	+1.84	+2.23	+1.01

CM 60 = Chiang Mai 60, Differences = Mutant minus Original

Results

No selection was made in the M_1 generation. The M_2 seed was bulked and M_2 plants with good agronomic traits were selected by comparison

with the original parents. The average seed protein content of the M_2 generation, mutant lines of Chiang Mai 60, SSRSN 35-19-4 and EHP 275 were 45.2%, 44.9% and 44.8%, while the original parents were 43.0%, 42.3% and 42.9%, respectively. M_3 plants were grown in rows. In each row, the best four plants were selected for protein analysis. Average protein content of mutant lines were 43.9%, 45.2% and 43.3%, while the three check varieties had average protein content of 42.4, 42.8 and 41.5%, respectively. In the M_4 generation, the mutant varieties had 0.78–2.23% higher seed protein content than the three checks. In a preliminary trial, the high protein mutant lines were tested for their protein yield. The mutants had average protein content of 42.5%, 42.4% and 42.9%. The check varieties had an average protein content of 41.8%, 40.3% and 41.9%, respectively (**Table 1**). The promising mutants selected from Chiang Mai 60, SSRSN35-19-4 and EHP 275 had six, 18 and eight lines. The mutant lines produced both high seed protein content and high yield. They will be further tested in replicated trials in research centers and farmers' fields.

Experiment II

Materials and Methods

CM9238-54-1 (ST) is a promising soybean line to be released to farmers. It gave 5–10% higher grain yield than Chiang Mai 60, the most popular variety in the northern and central regions. However, this line is susceptible to Soybean Crinkle Leaf (SCL) Disease. Approximately 5,000 seeds of the line with uniform size were irradiated with a dose of 200Gy Gamma-rays, as recommended by IAEA [3], at Kasetsart University. The M_1 plants were grown in the dry season of 2003 at Sukhothai Technical and Production Resources Service Center (TPRSC). The M_2 and M_3 seed were sown in dry and rainy seasons of 2004, and the selected M_4 lines were tested for grain yield in four environments: dry and rainy season of 2005, rainy season of 2006 and dry season of 2007 at Lop Buri TPRSC. The six mutants from the rainy season of 2006 and dry season of 2007 trials were tested for their protein contents. The Kjeldahl method [1] was used to determine seed protein percentages. For quality assurance of the data, duplicated samples, laboratory-fortified matrix samples, and an internal reference, soybean seed samples were also analyzed and included in each batch of samples. The protein content was calculated as the total nitrogen (N) content multiplied by 6.25.

Results

In the rainy season of 2006 and dry season of 2007 trials, the six selected lines were resistant to SCL in laboratory tests and yielded 74–81% more grain than the original parent. In addition, the six mutants had 2.1–4.0% and 2.1–7.5% higher seed protein content than a check variety, Chiang Mai 60, respectively, and had 0.5–2.0% and -1.0–3.3% higher seed protein content than another check variety, SJ4, respectively. The six mutants had average protein content of 38.5–43.8% while the two check varieties had average protein content of 36.3–39.9% (**Table 2**). The six mutant lines will be tested in the farmers' fields.

Discussion

It is possible to select mutant lines with greater yields and seed protein contents one to two percent higher than their parents. Soy protein is an important source of high-quality, cholesterol-free, inexpensive protein. It is suited to human physiological needs and has great potential as a major source of dietary protein for the future. The policy of the Thai government is to increase grain protein percentage for day food products. In the international marketplace, buyers pay six to 15 cents per bushel more for soybeans with just 1% higher protein content. The results of two studies presented here, suggest that soybean variety improvement for increased nutritional quality protein content is possible using induced mutation techniques.

Table 2. The seed protein content of mutant varieties, rainy season of 2006 and dry season of 2007 at Lop Buri Technical and Production Resources Service Center.

Varieties	Seed protein (%)					
	Rainy 2006			Dry 2007		
	Protein (%)	Dif.CM 60	Dif.SJ 4	Protein (%)	Dif.CM 60	Dif.SJ 4
POP1-4–Mutant	40.78	+4.07	+1.99	42.86	+6.51	+2.88
POP16-42–Mutant	40.15	+3.44	+1.36	43.29	+6.94	+3.31
POP18-46–Mutant	40.23	+3.52	+1.44	38.45	+2.10	-1.53
POP19-49–Mutant	39.89	+3.18	+1.10	38.62	+2.27	-1.36
POP20-50–Mutant	40.70	+3.99	+1.91	43.84	+7.49	+3.86
POP30-1–Mutant	39.26	+2.55	+0.47	39.04	+2.69	-0.94
CM9238-54-1(ST)–Original	38.79	+2.08	0	39.04	+2.69	-0.94
SJ4–Check	38.79	+2.08	0	39.98	+3.63	0
Chiang Mai 60–Check	36.71	0	-2.08	36.35	0	-3.63

CM 60 = Chiang Mai 60, Dif. = Differences of Mutant minus Check

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Genetic Enhancement of Speciality Rice through Induced Mutation – Short-Grain Aromatic Rice

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Abstract

India has a rich diversity of widely distributed aromatic rices. They include Basmati, whose cultivation is limited to a well-demarcated geographic zone, and the short grain aromatic rices that are grown in localized pockets throughout India, which with their unique sensory and cooking traits, cater to defined groups of consumers in specific niche markets. Some short-grain aromatic rices like Dubraj, Durgabhog, Makarkanda, Badshabhog are superior to Basmati in traits like high kernel elongation, high volume expansion and high head rice recovery, while Bindli is superior to Basmati in aroma and grain elongation. Kalajeera is known for the retention of aroma even after long storage. To date, little attention has been paid to short-grain aromatic rice, as the focus was directed towards improvement of Basmati. Less attention to the short-grain aromatic rices can be attributed to their low productivity, long duration and tall plant stature. As high economic returns are feasible with high-yielding varieties with shorter duration and shorter height, a mutagenesis approach was attempted to induce erect, semi dwarf, non-lodging mutants with high yield potential, while keeping the unique grain type and cooking quality traits of the parent cultivar. Twelve popular cultivars from different states (Kalanamak, Dubraj, Tulsiphool, Randhunipagal, Badshahbhog, Katrani, Improved Raskadam, Kalajeera, Pimpudibasa, Chinikamini, Dhusara and Kalajoha) were subjected to gamma (γ) irradiation. From the M_2 generation, selection was initiated to isolate mutants with shorter duration and plant height. Mutants with shorter (~20%) stature derived from Kalanamak, Dubraj, Kalajeera and Chinikamini showed high promise in the evaluation trials. Mutants with shortened duration (~10d) were also isolated in all the four genotypes. With the isolation of mutants with desirable traits, an expansion in the area under short grain aromatic rices is feasible, and the expected rice surplus can augment farmers' income, as well as Indian exports.

Introduction

Indian aromatic rice germplasm is well known because of Basmati, a national heritage, which occupies most of the area under aromatic rice. This is complemented by a large number of aromatic short and medium grain land races, grown in specific pockets in different states of the country [1]. These specialty rices are endowed with quality characteristics that are not present in ordinary rices [2]. These rices are equal to, or superior to Basmati, having strong aroma and greater kernel elongation [3], the only difference being breadth wise swelling and shorter kernel length. These aromatic rices of the Indian subcontinent are classified in Group V along with Basmati [4]. Most of these land races are photosensitive, tall, late maturing, low yielders, and distributed in localized small pockets throughout the country. Most research efforts have concentrated on the improvement of Basmati rices as they are linked with valuable foreign exchange, and native short grain aromatic rices could not attract the desired attention [5]. With the country gaining self-sufficiency in rice and transforming itself from a net importer to the second largest exporter of rice in the world, vast scope exists for these high value speciality rices that are famous for their cooking and eating qualities. Though Basmati's share is intact in the market, with the improving economic standards of the people, enough opportunities are available for commercial exploitation of these rices. Keeping this in mind, efforts are being made to improve the native land races through alterations in a few characters, such as duration and plant height, while retaining the unique quality features of each variety. A few aromatic short-grain varieties have been released in different states, including Ambemohar mutant in Maharashtra [6]. As it is a necessary to keep grain quality traits intact, it is difficult to get recombinants with the desired traits through pedigree breeding, but an induced mutation approach is ideal.

Materials and Methods

Seeds from 12 popular cultivars (Kalanamak, Dubraj, Tulsiphool, Randhunipagal, Badshahbhog, Katrani, Improved Raskadam, Kalajeera, Pimpudibasa, Chinikamini, Dhusara and Kalajoha) were treated with with three doses (200, 250 and 300Gy of ^{60}Co γ-rays at Utkal University, Bhubaneswar, in 2004. The irradiated seeds were sown in trays and transplanted to field with 10x10cm spacing. The fertilizer input was minimal to discourage tillering. The percentage survival was recorded in the M_1 generation and panicle-to-row progenies were grown in subsequent generations. The frequency of chlorophyll mutants in the M_2 generation was recorded as a ratio of panicles segregating chlorophyll mutations to the total number of panicles sown. Since all the cultivars were photoperiod sensitive, advancement of generations was undertaken only during the wet season, and starting from M_2, the main criteria for selection was erect, non-lodging plant habit and earliness, while efforts were made to keep the parental grain characters intact. Promising mutants were evaluated in both M_3 and M4 generations in observational trials conducted at the CRRI experimental farm.

Results and Discussion

The irradiated seeds of 12 cultivars were raised in trays and later transplanted in the field at very close spacing and the first three panicles from each individual M_1 plant were collected [7]. The materials were checked for treatment effects by observing seedling height using the growing rack method [8] that can effectively distinguish the effects of radiation-like delayed germination, stunted growth, poor vegetative vigor etc. (**Fig. 1**).

The results of four genotypes, Chinikamini, Dubraj, Kalajeera and Kalanamak, are presented in Table 1. A minimum of 200 plants (two treatments, 250 and 300Gy, of 200 seeds each) were raised in the M1 generation, and more than 600 lines (400 M_1 plants x 3 panicles each) for each treatment were grown in the M_2 generation. The frequency of chlorophyll-deficient mutations were scored (**Table 2**).

The data shows that genotypic differences exist in the biological damage caused by the radiation treatment. The chlorophyll-based mutation frequency was highest (23.8%) in Dubraj while the lowest (~8%)

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **197-199**

Figure 1 Screening of mutagen treated populations (M_1 generation) through seedling rack method.

damage was seen with Kalajeera. However, the chlorophyll mutation frequency was found to have no relation to the other traits under study, as the frequency of lines with the desired alterations, i.e. short duration and shorter height, were higher in Kalajeera than in Dubraj and Kalanamak.

These four short-grain aromatic rices have excellent grain and cooking quality characteristics (**Table 3**), and while making selections, it was ensured that these quality traits remained intact.

In the M_3 generation, selections were made exclusively on the shorter plant height and the results are presented in **Table 4**. The yields were calculated on a single plant basis.

Table 1. Number of plants grown from treated populations and mutants isolated in different generations

Genotype	M_1*	M_2**	M_3	M_4
Kalajeera	400	1169	98	15
Kalanamak	400	1173	22	9
Chinikamini	400	1130	66	19
Dubraj	400	1118	25	18

* @ 200 plants for each treatment
** @ Progeny from around 600 first formed panicles from each of three M_1 plant

Table 2. Frequency of chlorophyll deficient mutants in different genotypes.

S. no	Cultivar	No of M_1 plants grown	No M_2 lines segregating for chlorophyll mutations	(%)
	250Gy			
1.	Kalajeera	530	43	8.11
2.	Chinikamini	528	69	13.06
3.	Dubraj	541	81	14.97
4.	Kalanamak (37632)	534	87	16.29
	300Gy			
1.	Kalajeera	569	48	8.43
2.	Chinikamini	545	109	20.00
3.	Dubraj	589	140	23.76
4.	Kalanamak (37632)	584	115	19.69

Table 4. The range of the different characters observed in mutants with reduced plant height in different genotypes in the M_3 generation.

Parent and mutants	Plant height (cm)	Panicle length (cm)
Chinikamini		
Putative Mutants (66)	129-157	28.5
Parent	161	24
Dubraj		
Putative Mutants (25)	100-146.6	20.9-26.6
Parent	157.5	23.8
Kalajeera		
Putative Mutants (98)	141-151	28.2-32.6
Parent	176.5	32.5
Kalanamak		
Putative Mutants (22)	122-135	22.5-24.2
Parent	176.5	32.5

The yield did not appear to increase, although mutants with significant reduction in height were isolated in each genotype. Mutants with semi-dwarf stature (~100cm) were observed only in Dubraj, with a reduction of more than 50cm in height, while in Kalajeera the reduction in plant height was only 30cm, with mutants in the other two cultivars having intermediate heights (**Fig. 2**).

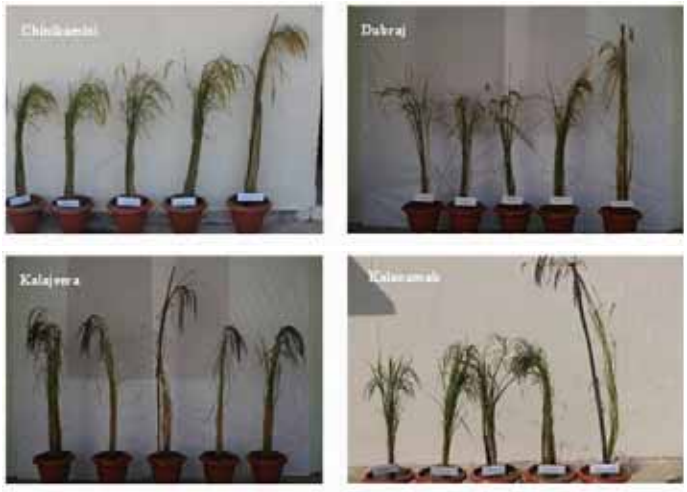

Figure 2 Mutants with reduced plant height induced in different short grain aromatic rice cultivars (the highest plant in each photo is the parent control)

In the M_4 generation, some of the mutant lines showed high levels of uniformity. Observations were made, except for yield potential of the mutant lines, and selections were made for short duration and plant yield, while trying to keep the grain quality intact (**Table 5**). Eighteen

Table 3. The physico-chemical properties of grain quality of aromatic rices

Genotype	MILL (%)	HRR (%)	KL (mm)	KB (mm)	L/B Ratio	GrnTy	Grain chalk	VER	WU (ml)	KLAC (mm)	ER	ASV	AC (%)	GC mm
Chinikamini	69.4	67.2	3.43	2.17	1.58	SB	VOC	5.3	175	6.8	1.98	5.0	23.30	53
Dubraj	70.2	56.7	5.83	1.94	3.0	MS	voc	4.9	130	10	1.71	7.0	25.86	48
Kalajeera	67.5	60.7	3.95	2.12	1.86	SB	VOC	5.3	187	8.0	2.01	4.7	22.68	63
Kalanamak	64.9	51.8	5.46	1.92	2.84	MS	voc	5.3	195	9.9	1.81	5.0	25.52	53

Source:Directorate of Rice Research Annual Progress Report, Vol 1(2003-2005). Mill: milled rice; HRR: head rice recovery; KL: kernel length; KB: kernel breadth; Grn Ty: grain type; VER:volume expansion ratio;WU:water uptake;KLAC:kernel length after cooking:ER;elongation ratio;ASV:alkali spreading value;AC:amylose content;GC:gel consistency

desirable selections of Dubraj were advanced to the M_4 generation, but the crop was severely affected by rice tungro disease, which led to stunted growth of several mutant lines, and as a result, no observations were recorded and the generation will be grown again.

Table 5. The range of the different characters observed in mutants with reduced plant height in different genotypes in the M_4 generation.

Parent and mutants	DFF	Plant height (cm)	Panicle length (cm)	Harvest index	Yield (kg/ ha)
Chinikamini					
Mutants (19)	110-120	122-156.4	20.3-23.9	0.19-0.42	1044-2698
Parent	120	168	22.6	0.31	2642
Kalajeera					
Mutants (15)	115-123	141-146	28.2-33	0.10-0.18	
Parent	121	176	30.6	0.11	
Kalanamak					
Mutants(9)	110-130	122-150	22.5-24.2	1380-2152	
Parent	131	176	32.5	2560	

DFF: Days from sowing to 50% flowering.

Discussion

The data clearly indicates that mutants with shorter duration could be induced in four genetic backgrounds, while reductions in plant height varied from genotype to genotype. Plant height varied from 122cm to 156cm in the mutant lines, and for duration, a 10-day reduction was seen. However, with the reduction in plant height, yield levels also declined and efforts are now underway to grow more plants from each genotype. Selections will be based on duration, plant height and yield. As expressed in several quarters, there is increasing demand to develop a market for these short-grain aromatic rices. Mutants with shorter plant stature with a high response to fertilizer are needed to exploit the enormous diversity of aromatic rice germplasm [9]. These rices could constitute a third distinct category of rices, after Basmati and non-aromatic rices for export purposes.

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Development of a High Oleic Soybean Mutant and its Stability Across the Environments

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Abstract

Modifying seed oil composition has become a major goal in soybean breeding programmes. Elevated oleic acid and reduced linoleic and linolenic acid content can improve the oxidative stability, flavor and nutritional value of soybean oil. The objectives of this study were (1) to develop a high oleic acid soybean mutant and (2) to determine the stability of its fatty acid composition across different environments. A high oleic acid mutant (HOM), containing 40% of oleic acid compared to 27% in parent cultivar MACS 450, was selected from a treatment with 200Gy γ-rays and 0.15% ethyl methane sulphonate (EMS). To investigate the influence of environmental factors on fatty acid composition, the HOM, along with four other soybean lines (MACS 1034, MACS 1055, MACS 1092 and Bragg) was grown at 12 locations. Seeds of each genotype from each location were analyzed for fatty acid composition by gas chromatography. Eberhart and Russell's linear regression model was used to study the environmental stability of fatty acid composition. In general, all the fatty acids studied were influenced by environmental factors. Elevated oleic acid in the HOM was less stable across environments than the oleic acid content in the other four cultivars. The mean oleic acid content in the HOM was 31.26 - 45.18% over the 12 locations. Linoleic acid content in the HOM and in MACS 1034 also showed significant deviation from unity for their regression coefficient, indicating significant environmental effects. This study shows that extent of the elevation of oleic acid and reduction of linoleic acid content in the HOM are strongly influenced by environmental factors.

Introduction

The end use of soybean oil is influenced by its fatty acid composition. Common soybean cultivars consist of 11% palmitic, 4% stearic, 24% oleic, 54% linoleic and 7% linolenic acid. Changing the proportion of these fatty acids will enhance food, fuel and other applications of the oil. The high content of polyunsaturated fatty acids (i.e. linoleic and linolenic) limits the utility of soybean as cooking oil, unless it is hydrogenated. Genetic modification of soybean oil composition to reduce polyunsaturated fatty acids and increase monounsaturated (oleic) fatty acids could be a viable strategy to improve oil stability and flavor without the need for hydrogenation, which produces undesirable trans fat causing increased cholesterol and heart disease in humans [1]. Also oil with a high content of monounsaturated fatty acids is less susceptible to oxidative changes. Mutation breeding is one of the best and widely used tools to alter the fatty acid composition in most of the oilseed crops [2-6].

Environmental influences on the fatty acid profile of soybean oil from common cultivars have been observed in several studies. The effects of year or location on the fatty acid content of soybean lines with different fatty acid profiles have been investigated in many reports [7, 8]. The differences in fatty acid content are likely to be a consequence of the different weather patterns, from year to year and location to location. Seed development at higher temperatures resulted in a significant decrease in linoleic and linoleinc acid and increase in oleic acid content [9-11]. Palmitic and stearic acids are generally unaffected by changes in temperature. Evaluation of stability of oleic, linoleic and linolenic acid contents of genotypes with modified fatty acid profiles is necessary to determine their utility in plant breeding programmes, developing soybean cultivars with enhanced oil quality and adaptation to a wide range of environments. This study had the objectives of developing a high oleic acid soybean mutant and determining the stability of its fatty acid composition across different environments.

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Materials and Methods

A high oleic acid soybean mutant (HOM), cultivar Bragg, and three soybean breeding lines (MACS 1034, MACS 1055, MACS 1092) were used in this study.

Mutation treatment

Seeds of soybean cultivar MACS 450 were treated with doses of γ-radiation and various concentrations of ethyl methane sulfonate (EMS). The mutation treatments given to seeds of MACS 450 are described by Patil, *et al.* [12]. The seeds of each treatment were sown in two rows. Each row consisted of 100 seeds with a distance of 5cm within rows and 45cm between rows. At the M_1 generation, 92 morphological variants were identified in the field and advanced until the M_4 generation by single seed descent. A line containing 40% oleic acid (as compared to 27% in MACS 450), derived from a treatment of 200Gy γ-radiation and 0.15% EMS, was selected from the M_4 population. Stability of the high oleic acid mutant (HOM) was tested until the M_8 generation at the research farm of the Agharkar Research Institute (Hol, Pune, India).

G X E interaction

To study the influence of the environment on fatty acid composition, the HOM, four soybean lines and cultivar Bragg were grown at 12 locations in the rainy season of 2006. Locations, latitude, planting dates and soil types are listed in **Table 1**. The trial was conducted in a randomized complete block design, with three replications at each location. Each row was three meters long. Distance between the rows was 45cm. Planting was done by hand with a seeding rate of 100 seeds per row.

Fatty acid analysis

An approximately 20g seed from each sample was ground in a mill. Out of this, approximately 200mg of powder was taken for fatty acid extraction. The fatty acid extraction was carried out according to the method of Primomo, *et al.* [13]. Fatty acid analysis was carried out on a gas chromatograph (6890 N series, Agilent Technologies Inc., Wilmington, DE, USA) using a HP-Innowax capillary column (J&W Scientific, Agilent Technologies Inc., Wilmington, DE, USA). The temperatures of injector, oven and detector were adjusted to 225°C, 150°C and 275°C, respectively. The initial oven temperature of 150°C was ramped by 15°C/min up to

Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **200-202**

250°C. The air, hydrogen and nitrogen (carrier gas) flow rates were set to 400 mL/min, 30 mL/min and 2 mL/min, respectively. Methyl esters of palmitic, stearic, oleic, linoleic and linolenic acids (Sigma Chemical Co., St. Louis, MO, USA), were used as standards to calibrate the method. The signals from the detector were integrated as normalized percentages from the calibration curve by using the HP CHEMSTATION software (Agilent Technologies Inc., Wilmington, DE, USA).

Table 1. Latitude, soil type, planting dates for each of twelve locations, 2006

Sr No	Location	Soil Type	Planting dates	Latitude (°N)
1	Amlaha	Black	6 July 2006	23.12
2	Amravati	Medium Black	7 July 2006	21.¬89
3	Bangalore	Red Sandy Loam	27 July 2006	12.58
4	Dharwad	Black	26 June 2006	15.26
5	Jabalpur	Clay	2 July 2006	23.90
6	Jalna	Black	26 June 2006	19.52
7	Nagpur	Clay	3 July 2006	21.09
8	Pachora	Medium Black	30 June 2006	20.83
9	Pantnagar	Sandy Loam	26 June 2006	29.00
10	Pune	Clay Loam	22 July 2006	18.04
11	Ranchi	Red Latteritic	27 June 2006	23.17
12	Sangli	Clay	11 July 2006	16.08

Statistical Analysis

Analysis of variance (ANOVA) for each location and a combined ANOVA across the test environments was performed for all fatty acids. To characterize the genotypic stability for fatty acid composition, Eberhart and Russell's (1966) linear regression model was used. For each genotype, stability was described by three parameters viz., mean performance (X), regression from the mean performance on environmental index (bi) and the function of squared deviation from regression (S^2_{di}). Combined ANOVA and stability analysis was performed using Agrobase/4TM (Agronomix Software Inc., Manitoba, Canada).

Results and Discussion

Development of the high oleic acid soybean mutant (HOM)

As result of mutagenic treatments, 92 morphological variants, including heterophylly, late maturing, early maturing, high-yielding and white-flowered were observed in the M_2 generation in the field. Each was harvested individually and advanced by single seed descent to the M_3 generation. Fatty acid analysis of seed from these mutants showed a wide range of variation for oleic acid content (23-40%). A high oleic acid mutant (HOM) from a mutagenic dose of 200Gy γ-radiation and 0.15% EMS was selected. Its seed contained 40% oleic acid whereas the

Table 2. Pooled ANOVA

Source	D.F.	Mean Squares				
		Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Genotype	04	3.29^{ab}	0.25^{ab}	511.98^{ab}	565.65^{ab}	0.61
Environment + (Genotype x Environment)	55	0.39^{a}	0.08	10.55^{ab}	6.37^{ab}	0.60^{a}
Environment (linear)	01	8.90	1.31	301.2	350.2	15.4
Genotype x Environment (linear)	04	0.41^{ab}	0.05^{ab}	7.85^{ab}	4.71^{ab}	0.15^{ab}
Pooled deviation	50	0.22^{ab}	0.05^{ab}	4.96^{ab}	2.51^{ab}	0.33^{ab}
Pooled error	120	0.02	0.001	0.03	0.035	0.01

$^{a, ab}$ Significant at probabilities of 0.05, 0.01, respectively

Table 3. Mean seed oleic acid content (%) over 12 locations[c]

Variety	1	2	3	4	5	6	7	8	9	10	11	12	Mean
MACS1034	20.6	20.0	21.9	21.4	27.2	23.5	27.5	21.4	23.6	21.2	19.2	25.4	22.8
HOM	34.2	34.7	31.3	35.7	44.0	32.9	40.6	40.4	40.4	39.4	34.7	45.2	37.9
MACS1055	21.8	18.6	27.8	21.3	24.4	23.4	25.0	19.8	24.3	22.9	19.6	22.2	22.6
MACS1092	24.3	23.8	25.6	24.5	29.8	24.5	22.2	25.3	24.8	23.6	21.8	32.2	25.2
BRAGG	18.2	18.4	24.2	23.5	20.5	22.6	27.4	24.9	24.6	22.7	21.6	28.0	23.0
Mean	23.8	23.1	26.2	25.3	29.2	25.4	28.5	26.4	27.5	26.0	23.4	30.6	
Ij	-2.5	-3.2	-0.1	-1.0	2.9	-0.9	2.3	0.1	1.3	-0.3	-2.9	4.3	
SE±	0.22	0.04	0.08	0.15	0.06	0.08	0.11	0.12	0.35	0.34	0.04	0.06	
LSD (P=0.05)	0.73	0.13	0.26	0.16	0.21	0.25	0.37	0.38	1.14	1.11	0.15	0.18	
LSD (P=0.01)	1.06	0.19	0.38	0.23	0.30	0.36	0.54	0.55	1.66	1.61	0.21	0.26	

[c] Columns entitled 1-12 in Table 3, 4 and 5 are the 3 locations described in Table 1.

Table 4. Mean seed linoleic acid content (%) over 12 locations[c]

Variety	1	2	3	4	5	6	7	8	9	10	11	12	Mean
MACS1034	57.3	55.4	54.7	55.8	52.5	54.6	52.2	55.8	54.4	56.7	57.3	52.8	55.0
HOM	43.6	42.1	43.0	39.8	34.8	43.0	38.9	37.0	37.9	37.7	41.4	33.0	39.4
MACS1055	57.2	58.3	50.6	55.7	53.6	55.4	53.7	57.5	53.8	55.1	57.4	55.8	55.4
MACS1092	54.9	54.1	51.9	53.0	49.7	54.4	54.7	53.0	53.4	54.1	55.2	47.1	53.0
BRAGG	60.3	56.4	54.1	53.3	54.7	55.7	52.3	53.6	54.9	57.1	56.7	50.4	55.0
Mean	54.7	53.3	50.9	51.5	49.1	52.6	50.4	51.4	50.9	52.1	53.6	47.8	
Ij	3.2	1.8	-0.6	0.003	-2.5	1.1	-1.1	-0.1	-0.7	0.6	2.1	-3.7	
SE+	0.14	0.03	0.06	0.13	0.06	0.16	0.08	0.10	0.49	0.36	0.14	0.05	
LSD (P=0.05)	0.44	0.11	0.20	0.41	0.21	0.52	0.26	0.33	1.59	1.18	0.46	0.15	
LSD (P=0.01)	0.64	0.16	0.30	0.60	0.30	0.76	0.38	0.48	2.31	1.72	0.66	0.22	

[c] Columns entitled 1-12 are the 3 locations described in Table 1.

Table 5. Mean seed linolenic acid content (%) over 12 locations[c]

Variety	1	2	3	4	5	6	7	8	9	10	11	12	Mean
MACS1034	7.9	8.1	8.7	8.7	7.0	7.4	6.9	7.6	7.4	7.7	8.6	8.3	7.9
HOM	6.7	7.5	8.7	8.5	7.0	7.8	6.4	6.9	7.0	7.4	8.2	7.3	7.5
MACS1055	7.2	8.1	7.1	8.7	7.1	6.8	6.9	7.9	7.1	7.7	8.4	8.2	7.6
MACS1092	6.8	7.0	7.9	8.0	6.5	6.5	8.2	6.8	6.6	7.6	8.3	6.9	7.3
BRAGG	7.5	9.0	7.7	8.8	9.3	8.0	6.3	7.0	6.6	6.0	8.0	7.9	7.7
Mean	7.2	8.0	8.0	8.6	7.4	7.3	6.9	7.2	7.0	7.3	8.3	7.7	
Ij	-0.3	0.4	0.4	1.0	-0.2	-0.3	-0.6	-0.4	-0.6	-0.3	0.7	0.2	
SE+	0.08	0.02	0.03	0.06	0.06	0.02	0.03	0.07	0.22	0.20	0.03	0.05	
LSD (P=0.05)	0.27	0.08	0.10	0.19	0.19	0.07	0.11	0.21	0.70	0.64	0.10	0.17	
LSD (P=0.01)	0.39	0.12	0.16	0.28	0.27	0.10	0.15	0.31	1.02	0.93	0.14	0.25	

[c] Columns entitled 1-12 are the 3 locations described in Table 1.

Table 6. Stability parameters for all fatty acids

Sr no	Variety	Palmitic acid			Stearic acid			Oleic acid			Linoleic acid			Linolenic acid		
		X	bi	S^2_{di}	X	bi	S^2_{di}	X	bi	S^2_{di}	X	bi	S^2_{di}	X	bi	S^2_{di}
1	MACS 1034	11.5	1.7	0.1^{ab}	3.0	0.8	0.03^{ab}	22.8	1.0	1.91^{ab}	55.0	0.8^{ab}	1.0^{ab}	7.9	1.1^{a}	0.1^{ab}
2	HOM	12.6	1.2	0.6^{ab}	2.8	0.5	0.02^{ab}	37.8	1.6^{ab}	7.31^{ab}	39.4	1.5^{ab}	4.1^{ab}	7.5	1.1^{a}	0.2^{ab}
3	MACS 1055	11.3	0.4	0.1^{ab}	3.1	1.1	0.01v	22.6	0.6	5.55^{ab}	55.4	0.6	3.8^{ab}	7.6	0.9	0.2^{ab}
4	MACS 1092	11.8	0.8	0.02^{a}	2.8	0.9	0.07^{ab}	25.2	0.9	4.79^{ab}	53.0	1.1	1.7^{ab}	7.3	0.7	0.4^{ab}
5	BRAGG	11.3	0.9	0.3^{ab}	3.1	1.7	0.14^{ab}	23.0	0.9	5.08^{ab}	55.0	1.1^{ab}	1.9^{ab}	7.7	1.2	0.8^{ab}

$^{a, ab}$ Significant at probabilities of 0.05, 0.01, respectively

control MACS 450 had 27% oleic acid. The HOM was advanced and multiplied until the M_6 generation. The stability of the high oleic acid trait was tested at each generation at Hol Farm, Pune. It showed 34% to 67% oleic acid content in different generations. At the M_7 generation, in a replicated evaluation trial, analysis of variance showed a highly significant increase in oleic and decrease in linoleic acid content (P = 0.01; data not shown), whereas other fatty acids showed no significant change. Two high oleic mutants obtained by X-ray irradiation, M_{23} (46.1%) and M_{11} (35.9%), have been reported previously [15, 16]. The elevated oleic and reduced linoleic acid content in the HOM might be due to the mutation in seed specific, *fad2*-1 gene or genes [17], which code for a microsomal omega 6 desaturase enzyme. Also, HOM oil can have more oxidative stability as compared to MACS 450, as the rate of oxidation of linolenic, linoleic and oleic acid is in the ratio of 21.6:10.3:1.

G X E interaction

Combined analysis of variance showed significant differences among genotypes for all fatty acids except for linolenic acid (**Table 2**).

Environment + (Genotype x Environment) effects were significant for the content of all fatty acids, except for stearic acid, and the Genotype x Environment (linear) effect was significant for all fatty acids, showing a strong influence of the environment on these traits. Studies conducted in controlled environments have shown that temperature [10] and precipitation [11] have a major impact on fatty acid levels in soybean seeds. Therefore, temperature and precipitation could be the underlying factors that contributed to the location effects observed in this study. Significant differences for unsaturated fatty acids i.e. oleic, linoleic and linolenic acid were observed among genotypes at all locations. Unsaturated fatty acid content (oleic, linoleic and linolenic acid), environment index (Ij), standard error and LSD (at P=0.05 and 0.01) for each genotype over all 12 locations are given in **Tables 3, 4 and 5**.

Seeds of HOM had 31.26 - 45.18% oleic acid with an average of 37.8%, and 32.97 - 43.61% linoleic acid with an average of 39.35% across the 12 environments. By contrast, a narrow range for content of these fatty acids was observed in other cultivars containing normal oleic acid (**Tables 3, 4**). This indicated a strong influence of environment on the content of these fatty acids in HOM.

Stability parameters studied by Eberhart and Russell's model for each fatty acid are listed in **Table 6**.

Mean square deviations from linear regression (S^2_{di}) were significantly different from zero for all traits under study for all genotypes, indicating significant responses to changes in environments. However, significant deviation from unity for regression of the mean performance on environmental index (bi) was observed only for unsaturated fatty acids in a few genotypes. Only elevated oleic acid in HOM showed highly significant (P = 0.01) difference in bi, indicating above average stability of the mutated trait across the 12 environments. Similarly, highly significant deviation from unity for bi was observed in MACS 1034, HOM and BRAGG for linoleic acid. For linolenic acid, significant (P = 0.05) deviation in bi was observed in only MACS 1034 and HOM. The significantly high bi (greater than one) for oleic, linoleic and linolenic acids observed in HOM indicates its above average stability for these traits, and thus the influence of environmental factors on unsaturated fatty acids. In the case of HOM, an increase in oleic acid content was observed in warmer locations and a decrease in oleic acid content was observed in cooler locations, suggesting an effect of temperature on the oleic acid content of HOM. This is in agreement with earlier reports [13, 18].

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Study of Tomato Lines with High Nutritive Quality

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Abstract

This study was performed on tomato lines from the cultivated species carrying the mutant genes *hp* and *og*^{c}, as well as on lines originating from inter-specific hybridizations between *Solanum lycopesicum* Mill. and either *Solanum pimpinellifolium* Mill. or *Solanum chilense* Dunal, to evaluate their genetic potential to synthesize high lycopene. We evaluated the methodology of comparison, and the number of harvests necessary to determine whether individual plants or lines differed in their ability to accumulate high lycopene in their fruit. A relatively large variation between harvest dates was observed in the lycopene content in fruit of the lines and hybrids studied, but the genotypes investigated were ranked almost identically at all harvests. It was found that the genotypes possessing genetic potential to synthesize high lycopene content might be assessed based on the analysis of fruit from a single harvest. Studies to fingerprint and evaluate DNA variability among tomato lines from diverse origins or possessing genes enhancing lycopene content, as well as on some of their F_1 hybrids, were performed. The AFLP data indicated very low levels of genetic heterogeneity in the tomato lines studied. Selective markers with a direct application in the molecular selection of tomato lines and hybrids with economically valuable mutant characters were revealed. The origins of the studied tomato lines make them genetically heterogeneous. Grouping performed on the basis of AFLP patterns followed the species origin of the genotype in most of the cases.

Introduction

Tomatoes are one of the most important crops in the world because of the volume of their consumption and overall contribution to human nutrition [1, 2]. They are as well, the major source of lycopene, an important phytonutrient for human health. Few genes that result in enhanced carotenoid accumulation in tomato have been identified. The *high pigment* (*hp*) mutation results in increased accumulation of both lycopene and β-carotene during fruit development. Mutant plants are characterized by elevated levels of anthocyanins and dark-green immature fruit, due to the overproduction of chlorophyll pigments [3]. Recent studies have provided evidence that *hp-1* and *hp-2* are separate loci, *hp-1* located on chromosome 2 and *hp-2* located on chromosome 1 [3, 4]. The practical use of these genes is rather limited because of their undesirable pleiotropic effects that include slow seed germination, increased seedling mortality and premature defoliation [5]. The crimson/old gold (*og*^{c}) gene increases lycopene by about 50%, while reducing β-carotene by the same amount [6]. Germplasm with enriched lycopene content (as well as enriched content of other compounds or characteristics related to fruit nutrition and flavor quality) might be developed on the basis of interspecific hybridization [7, 8]. However, the development of such germplasm is a difficult process, as the lycopene content of tomatoes depends not only on genetic factors, but also on environmental factors, such as a light intensity during ripening [9], temperature [10], growing season, location, irrigation, soil etc. [11, 12, 13, 14].

The present study was therefore designed to: 1) evaluate the methodology of comparison, as well as the number of harvests necessary to determine whether individual plants or lines possess the genetic potential to accumulate high lycopene content in their fruit, and 2) fingerprint and evaluate DNA variability among tomato lines from S*olanum lycopersicum* Mill. possessing economically important characters and differing in their lycopene content.

Materials and Methods

Plant Material

Studies were performed on the following genotypes: lines XXIV-13, St-993, C-19, B-317, VK-1 and G-32 [15, 16, 17]. In order to get a more reliable baseline for comparative evaluation of the variation in fruit lycopene content, Ailsa Craig (AC) and the near isogenic lines (NILs) differing in genes *hp* (*high pigment*) and *og*^{c} (*old gold -crimson*) described by [18] and their F_1 hybrids were included in the study.

Biochemical analyses

Ten fruit from each line or hybrid, of uniform size and color, were sampled at the same stage of maturity and used for biochemical analyses. Lycopene content was determined by chromatographic absorption [19] (mg %) and High Performance Liquid Chromatography (HPLC) (mg/100g dry matter). The analyses were executed in three replicates. The results are expressed as the means and standard errors calculated from replicates [20].

DNA extraction

Three or four unexpanded young leaflets were collected from individual plants. DNA was isolated using the PhytoPure Kit protocol (Amersham) using 0.033 g lyophilized leaves.

AFLP analysis

AFLPs were obtained according to the protocol of Kashkush, *et al.* [21] involving: (i) *Restriction* (using the enzymes *Eco*RI, *Mse*I) /*ligation* (of adapter pairs onto restriction sites ends), (ii) *Preselective amplification*, (iii) End labeling (with γ-ATP-P^{32} of *Eco*RI-based primers), (iv) Selective amplification (with selective primer pairs *Eco*RI/*Mse*I: 1-ACT/CAT, 2-ACA/CAT, 3-AGG/CTT, 4-ACT/CAG, 5-ACC/CAC, 6-CAG/CAG, 7-CGT/CAT), (v) *Electrophoresis* (using PAAG) and (vi) Scoring of markers: Those bands showing clear polymorphism among genotypes were scored. A matrix was designed based on the total number of bands in all studied samples for each selective primer pair. Individuals with presence of a band were given a score of "1". The lack of a band was given a score of "0".

Statistical analysis

Relative frequencies were calculated by a standard method. For expressing the genetic distances among AFLP patterns a cluster analysis method

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Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009,**203-206**

using Euclidian distances was applied, especially linkage distances, using the statistical package Statistica Version 6.0.

Results and Discussion

Table 1 shows that there was large variation between harvest dates in the lycopene content of fruits from the lines and hybrids studied. In all genotypes, fruit lycopene content increased from July 25th to August 10th to August 25th. Fruit lycopene content evaluated on August 25th exceeded that on July 25th, on average by 25%. According to Helyes, *et al.* [9] rainfall, temperature and light intensity during the period preceding the harvest date exercise a considerable effect on lycopene content. It is possible that such environmental factors influenced the fruit lycopene content on different harvest dates.

The genotypes could be divided into three groups based on their lycopene content: 1) genotypes that always have the highest lycopene content (G 32, Ailsa Craig *hp* and Ailsa Craig *og*^{c}), 2) genotypes always having low lycopene content (VK 1, B 317, C 19), and 3) genotypes with lower lycopene content than those from group 1, but significantly higher lycopene content than those from group 2 (XXIV 13, St 993, Ailsa Craig, Ailsa Craig *og*^{c} x Ailsa Craig *hp*). This data suggests that genotypes with genetic potential to synthesize high lycopene content might be identified from analyses of fruit collected at one harvest date. However, comparisons of genotypes sampled on different harvest dates could be misleading. A second, and if possible, a third analysis on different harvest dates might be helpful in providing additional information on a genotype's capacity to synthesize lycopene, as well as its variation during the harvest season.

Table 1. Variation in fruit lycopene content of tomato genotypes sampled on three harvest dates.

Lycopene								
Absorption chromatographic method / Rank (R) (mg%)							HPLC / Rank (R) (mg/100g dry matter)	
Genotype	July 25th	R	August 10th	R	August 25th	R	August 25th	R
G 32 *hp og*^{c}	8.6 ± 0.4	1	9.0 ± 0.3	1	10.9 ± 0.2	1	117.0 ± 0.2	2
A. Craig *hp*	6.7 ± 0.2	2	8.4 ± 0.3	2	8.9 ± 0.4	2	150.3 ± 0.4	1
A. Craig *og*^{c}	5.8 ± 0.2	4	7.4 ± 0.1	3	7.7 ± 0.3	4	92.8 ± 0.3	3
XXIV-13	4.8 ± 0.1	7	7.2 ± 0.2	4	7.8 ± 0.3	3	80.9 ± 0.2	4
AC *og*^{c} x AC *hp*	5.0 ± 0.3	6	6.9 ± 0.4	5	6.9 ± 0.1	6	75.4 ± 0.1	6
AC *hp* x AC *og*^{c}	5.6 ± 0.2	5	6.5 ± 0.2	6	7.2 ± 0.1	5	77.5 ± 0.1	5
A. Craig	4.7 ± 0.1	8	6.4 ± 0.4	7	6.8 ± 0.2	6	69.1 ± 0.2	8
VK 1	4.3 ± 0.1	9	4.5 ± 0.1	9	4.3 ± 0.1	9	51.3 ± 0.2	10
B 317	4.6 ± 0.2	8	5.0 ± 0.4	8	5.2 ± 0.3	7	54.1 ± 0.3	9
St 993	5.9 ± 0.3	3	6.4 ± 0.1	7	6.9 ± 0.2	6	70.4 ± 0.2	7
C 19	3.9 ± 0.1	10	4.2 ± 0.4	10	4.8 ± 0.1	8	50.4 ± 0.3	11

Fingerprinting and evaluation of DNA variability among tomato lines differing in their lycopene content were performed and data was summarized in **Figure 1**. Nineteen polymorphic bands were identified among 425 bands scored: ACT/CAT picked out C 19 and B 317 from the others of the two groups by the absence of band 10th in both genotypes. Two more bands associated C 19 and B 317 with each other, and were distinct from the other genotypes, with ACT/CAT. The ACA/CAT produced three polymorphic bands and was able to distinguish XXIV-13 from the other genotypes. This profile specificity was associated with the absence of two bands presented in the other members of the group (16 and 31), and band 30 that was not presented in the other lines. Five polymorphic bands were obtained with the combination AGG/CTT in three of the analyzed genotypes: XXIV-13, St 993 and VK 1. The compared profiles were completely identical after amplification with ACT/CAG and the genotypes were not distinguishable. Two polymorphic bands were found with ACC/CAC in three samples. They were different from the 10 others analyzed on the gels. C 19 could be distinguished from VK 1 and B 317 by one fragment. VK 1 and B 317 had identical DNA fingerprints. The four polymorphic bands obtained by ACC/CAC differentiated three from the others. Additionally, among the lines, one band separated B317 from VK1 and one band separated C 19 from B 317 and VK 1. A unique polymorphic band was found with CGT/CAT in VK 1 that was absent in the other samples. VK 1 and Ailsa Craig, considered as representatives of the cultivated species, showed comparatively more genetic divergence.

Ailsa Craig and St 993 composed one group according to the cluster analysis, and XXIV-13 was added to this cluster (**Fig. 2**). These three genotypes were classified in the same group based on their lycopene content (group 3). Both St 993 and XXIV-13 include in their pedigrees *S. pimpinellifolium* [13], which might explain their genetic similarity calculated on the basis of molecular markers. The other genotypes composed different clusters and their differences were significant. Both B 317 and C 19 were characterized by low lycopene content and both had *L. chilense* in their pedigrees [13, 15]. However, these genotypes were placed in separate clusters, close to each other. The finding that genotypes from group 2 and group 3 (based on their lycopene content) composed different clusters and is not useful for the identification of genotypes possessing high lycopene content, as this differentiation is due to species origin rather than to their lycopene content.

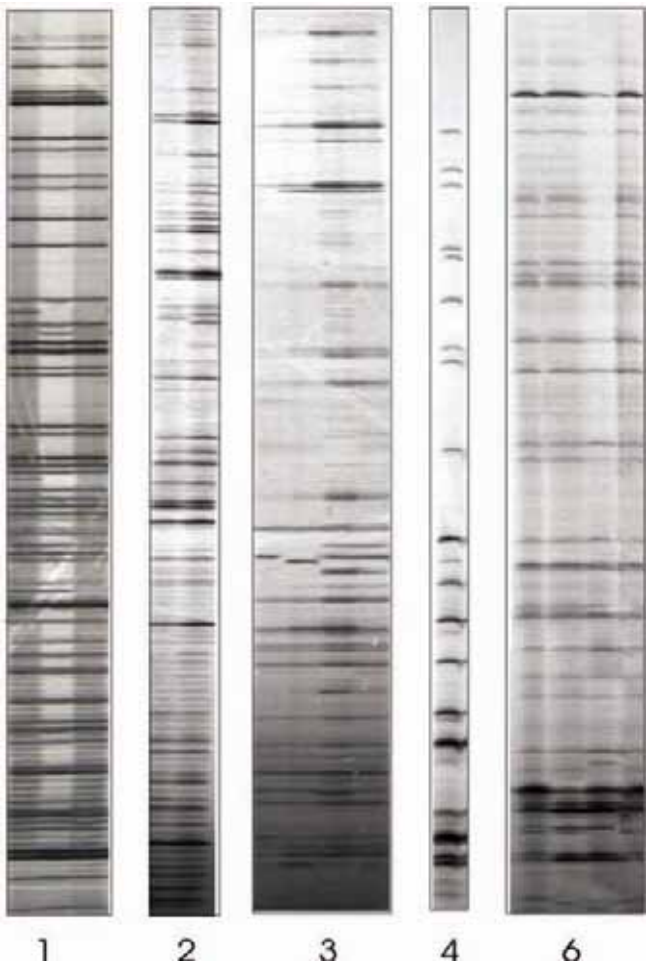

Figure 1 AFLP patterns of the genotypes: 1. ACT/CAT: 1.non polymorphic, 2.C19, 3.B317; 2. ACA/CAT: 1.XXIV-13, 2.non polymorphic lines; 3. AGG/CTT: 1.XXIV-13, 2.St993, 3.VK1, 4.non polymorphic; 4. ACT/CAG: non polymorphic; 5. ACC/CAC: not shown; 6. CGT/CAT: 3.VK1, 1., 2., 4.non polymorphic.

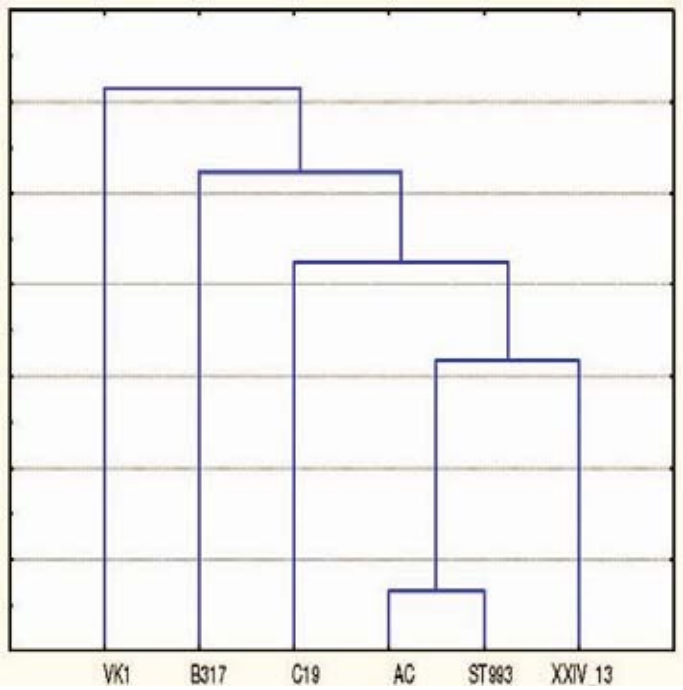

Figure 2 Clusters of AFLP patterns data from groups 2 and 3.

Conclusions

The ability of tomato lines to accumulate lycopene in their fruit was evaluated and this trait was found to be genotype-dependent. However, the accumulation of lycopene was observed to differ between harvests in all genotypes. Grouping performed on the basis of AFLP patterns followed the species origin of the genotype in most of the cases.

ACKNOWLEDGEMENTS

Work performed under Research Contract Nº12227 with FAO/IAEA within the CRP D23024 on Physical Mapping Technologies for the Identification and Characterization of Mutated Genes Contributing to Crop Quality. Thanks to Prof. Uri Lavi from Volcani Center – Israel for introducing the AFLP method applied for this study.

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