

## MOLECULAR GENETIC APPROACHES TO IMPROVE THE NUTRITIONAL QUALITY OF STAPLE FOOD CROPS

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### Summary

Plants are the main source of micro and macronutrients for most living organisms. Their capacity to photosynthesize allows them to transform solar energy into chemical energy stored in organic and inorganic molecules and use it for their own necessities. Animals, including humans, need plant nutrients, since many nutrients that are essential for the correct performance of vital animal functions are only synthesized by plants. For millions of years, humans have selected outstanding characteristics of crop plants like vigor, yield, flavor, resistance to pests and higher nutritional value; and nowadays the major cereals (rice, wheat, maize, barley, millet and sorghum) domesticated from wild relatives are the main source for human and animal nutrition. This selection has been made, based mainly on phenotypic characteristics, and successful progress has been somewhat slow. However, the human population has increased rapidly within the last hundred years and the need for appropriate foods, especially in developing countries is a priority for producers. The development of molecular biology offers the possibility for improving the desired characteristics of crop plants through the direct introduction of specific genes, with the advantage of being faster and more selective. Molecular biology also provides the tools for a complete molecular and physiological analysis of the

transformed plants. Genetic engineering is simply an additional tool for improving crop plants in combination with traditional breeding. Both strategies have been used in recent years to increase the nutrient content of some crop plants ensuring better quality foods for certain populations. This chapter presents a general view of the biotechnological methods currently used for improving plants and how they are being exploited to increase the content of specific nutrients in specific plant species important to the human population.

## 1. Introduction

Nutrition is the process through which organisms acquire and process chemical substances called nutrients to develop their basic vital functions, such as growth and reproduction. These nutrients may be obtained from air, water, soil or other organisms. Plants and cyanobacteria capture the energy of sunlight, and together with water and CO<sub>2</sub>, transform it to chemical energy by means of a succession of reactions collectively named photosynthesis. The final products of photosynthesis are energy-rich sugar molecules that constitute the substrates and energy source of a number of chemical reactions that enable plants to synthesize complex molecules, from metabolites to building blocks. Along with H and C (from H<sub>2</sub>O and CO<sub>2</sub>), another 15 elements are believed to be essential for the growth of most cultivated plant species. An element is judged essential if in its absence the plant cannot complete its life cycle. All 17 elements are needed in different amounts. Trace elements or micronutrients (Mo, Ni, Cu, Zn, Mn, B, Fe, Cl) are needed in tissue concentrations equal or less than 100 mg kg<sup>-1</sup> of dry matter, whereas macronutrients (S, P, Mg, Ca, K, N, O, C, H) are needed in concentrations of 1 000 mg kg<sup>-1</sup> of dry matter.

Since animals are unable to photosynthesize, they consume and process energy rich molecules from other organisms to obtain their nutrients. In order to maintain optimal health, animals including humans require the consumption of a set of macro and micronutrients. Animals therefore, depend on photosynthetic organisms either directly or indirectly to support their nutrition. Within this panorama, photosynthetic organisms are considered the chemical source of energy for life on earth, and plants, as photosynthetic organisms are essential for the survival of other non-photosynthetic species.

Animal macronutrients (carbohydrates, lipids and proteins) make up most of the bulk of foodstuff and are used primarily as an energy supply, whereas micronutrients (organic and inorganic compounds) are needed in small amounts and contrary to macronutrients, are not used as energy sources. Nonetheless, micronutrients are essential components of cells and tissues and are also required to accomplish physiological functions, such as muscle contraction and the conduction of nerve impulses. Essential micronutrients in the human diet include 17 minerals and 13 vitamins. Non-essential micronutrients encompass a vast group of unique organic phytochemicals that are not strictly required in the diet, but are linked to the promotion of good health. To prevent nutrient deficiencies in humans, each group of nutrients has to be consumed at a daily intake level above a defined minimum value. The level of requirement of each nutrient varies with age, sex and physiological status. Additionally, the intake of some nutrients at

higher, therapeutic levels has been associated with a reduction in risk for chronic conditions.

Plant components with nutritional significance or benefit for human health have been named phytonutrients. These plant-derived nutrients are not ready packaged in specific plants to satisfy human needs, they are distributed throughout a variety of plant species to satisfy the metabolic requirements of each species, furthermore each plant species is found naturally in a specific environment and not distributed worldwide. The perfect plant, to satisfy human needs in nutritional terms, does not exist. Moreover, modern agriculture has become highly specialized concentrating on a handful of staple crops namely: rice, maize, wheat, potatoes, sweet potatoes, and grain legumes. Cereal crops are rich in carbohydrates, providing more than 80% of calorie intake, and grain legumes rich in protein often replace meat in the diets of populations in developing countries. However, these crops are poor sources of some macronutrients and many micronutrients, which can lead to serious health problems in the human population. For this reason, traditional breeding efforts and more recently biotechnological strategies have focused on increasing the content of such nutrients in staple crops.

## **2. Crop Improvement**

Plant breeding has been the main strategy to improve the nutritional value of plants. Traditional plant breeding strategies are based on the knowledge obtained through the investigations of the inheritance of any character, the mode of inheritance, and existing genetic variability. Appropriate breeding strategies make use of phenotypic selection of individuals or families. The methods for selection first originated from the crossing between individuals of the same species or closely related species. The outstanding individuals (hybrids) are selected throughout several crossing and backcrossing cycles to finally obtain a generation carrying the desirable trait and which is also commercially acceptable: the new individuals are known as a new variety.

Traditional breeding has been enormously successful in producing new varieties adapted to different stresses, such as attack by pathogens and pests, or giving high yields. Unfortunately, the majority of breeding efforts are aimed at the needs of consumers in developed countries and not at improving crops for use in developing countries where environmental conditions, diseases and pests can often have devastating effects.

Despite their success, traditional breeding methods do have certain drawbacks. Often desirable traits are found in land races or other closely related species, therefore to introduce the character of interest, many rounds of subsequent selection are necessary to reduce the linkage drag or remove all the undesirable traits present in the non-commercial parent. Another drawback is the effect of the environment on the trait of interest. Therefore, breeders must distinguish between the real genetic potential of the plant and the effect the environment may have. Finally, simply producing inter-species crosses can be difficult, since they produce few viable seeds from which to select.

In part, these drawbacks are being alleviated by the increasing use of molecular markers associated with traits of interest. Selection can be made on the basis of molecular

marker genotype eliminating the problem of environmental effects and allowing selection on a genome-wide basis. Therefore, selection for the elimination of undesirable traits is more effective. Molecular markers are also important for the selection of complex traits where multiple genes are involved. As will be discussed below, marker assisted selection for complex traits such as yield, drought tolerance, etc., will be an essential tool in producing new varieties at least until the underlying genetic mechanisms governing these traits have been revealed.

## 2.1. Genetic Engineering

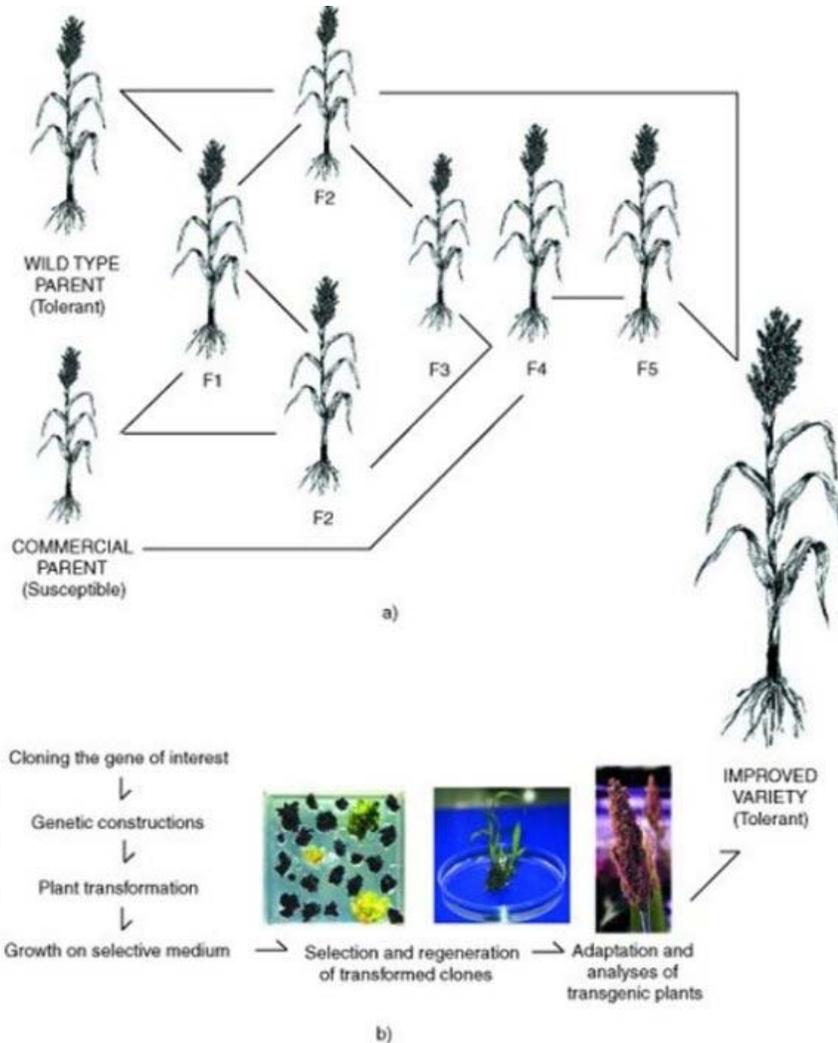


Figure 1. Strategies for the improvement of crops through traditional breeding (a), and genetic engineering (b).

Genetic engineering refers to sophisticated, artificial techniques capable of transferring genes from unrelated organisms directly to recipient organisms. Genes are the units of genetic information found in all organisms, and they contain regulatory elements that stimulate or silence their expression in a tissue and temporal specific manner. Genes

determine specific traits, like color, height, or tolerance to frost. Adding novel genes to crops means adding new traits and abilities. Genetic engineering allows the incorporation in plants of genetic information from any other organism, including bacteria, animals, insects, etc., in addition to plants.

The expression of different genes of an individual is coordinated by genetic regulatory elements (promoters, enhancers and silencers) that determine changes in growth, development, and cellular differentiation. Genetic engineering leads to direct access and manipulation of the information contained in DNA. It also permits the creation of synthetic genes.

Major advances in the transformation of plant species have come from the development and improvement of transformation technologies, plant tissue culture and regeneration from transformed cells or tissues, as well as basic research in plant molecular biology and physiology.

The modification of plant components using molecular genetic technology is aimed at increasing plant production, quality or quantity of products, lowering production costs and even the production of important biological molecules difficult to find or not found in plants or even in nature. Agricultural biotechnology, through genetic engineering, is making rapid progress, and transgenic plants are being introduced commercially at an increasing rate. Genetic engineering reduces the time taken to obtain an improved cultivar (see Figure 1). Details about the introduction of foreign genes into plant cells will be discussed in Section 2.1.2.

### **2.1.1. Identification of Genes with Potential to Improve the Nutritional Quality of Plants**

Techniques of molecular biology are employed to study the genes of different organisms. These strategies permit the study of specific components within the genome and the interrelationship of such components as a whole. Using these means, it has been possible for scientists to identify the gene or genes whose products participate in diverse metabolic pathways. The basis for genome analysis in plants is a genomic map, which can be either a genetic map based on information from both visible and molecular markers, or a physical map, in which yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs) are aligned with the chromosomes to give a position to genes within the genome.

A current approach to gene discovery that is most applicable to compounds of nutritional importance, synthesized or accumulated by plants and other organisms has been named nutritional genomics. This discipline takes advantage of the homologies or similarities between the metabolic routes that lead to the generation of a given product in different organisms. In this context, previously characterized genes from the metabolic pathway under research, either from animals, microorganisms or any plant species can be found in public databases, and may be used as a source of genetic information to study or modify the characteristics of the target plant. These modifications can be accomplished by the introduction of foreign genes isolated from

other species or by cloning genes from the recipient plant using as a molecular tool the information from the genes already isolated from other organisms.

An example of the application of nutritional genomics is the discovery and modification of genes involved in the synthesis of vitamin E in plants. The first gene involved in this pathway in *Arabidopsis thaliana* was isolated with fungal and human orthologs as database queries. The sequence of the *Arabidopsis* gene served to identify an ortholog in a 10-gene operon in the cyanobacterium *Synechocystis* PCC6803. Gene disruption experiments showed that this operon also encodes for a  $\gamma$ -tocopherol methyl-transferase ( $\gamma$ -TMT), which is the final step in vitamin E synthesis. This  $\gamma$ -TMT gene of *Synechocystis* allowed the isolation of an ortholog from the *Arabidopsis* database, whose over-expression increased vitamin E levels nine-fold in *Arabidopsis* seed oil.

When there is a lack of information on the specific gene or genes whose products are involved in the metabolic pathway of interest, the isolation of genes becomes more complicated, but researchers have developed alternative strategies. Some of these strategies have their basis in mutagenesis, or alterations of the genome that produce changes in the phenotype due to the modification of the components of a given metabolic route. The gene(s) modified through mutagenesis may be localized by molecular marker mapping techniques, and may be further isolated and characterized.

In maize, different mutants affected in seed development have been obtained, and the altered genes responsible for their phenotype have been cloned using mapping techniques. Such is the case of *miniature-1*, whose phenotype is grain size reduction, which is the result of the lack of extracellular invertase activity. A mutant named *shrunk* presents starchless grains and the affected gene was identified as the endosperm sucrose synthase.

Other advances have been made in the application of techniques to identify and isolate genes, including the sequencing of entire genomes of prokaryotic organisms. Recently, new approaches have been developed that allow the mutagenesis of large numbers of genes. These procedures are of two types: first, “random” insertional mutagenesis, in which insertion mutations are randomly generated throughout the genome, followed by the identification of the gene(s) affected by comparing the sequence adjacent to the insertions with the genome sequence, or expressed sequence tags (EST), and second, targeted mutagenesis in which specific genes are deleted or analyzed.

The use of transposons and retrotransposons has made it possible to carry out non-site directed mutagenesis. This approach is based on the features of natural transposable elements that are ubiquitously found in eukaryotic organisms and whose integration into a new location within the host genome can disrupt genes, effectively producing a “tagged” mutation. A similar approach is to use *Agrobacterium tumefaciens* T-DNA insertions to produce mutations.

The methods described above generally work on a “one gene in one experiment” basis, where the whole picture of gene function is hard to obtain. Nevertheless, other methods let scientists monitor a wider range of gene expression, and therefore form a more complete picture of gene expression and the interaction of gene products. Two-

dimensional electrophoresis and methods for large-scale analysis of proteome variations has evolved into “proteomics”, which can be used to map translated genes and loci controlling their expression, identifying variations of complex phenotypic traits.

Among other techniques, differential display is based on the analysis of differential mRNA populations produced in an organism as a result of its exposure to two or more different environmental conditions. In addition, large-scale DNA sequencing and microarrays are new technologies that promise to monitor the whole genome on a single chip, so that the interactions between thousands of genes can be analyzed simultaneously. These advanced methods can be subjected to a combined bioinformatic-based and expression-based analysis to identify a limited set of candidate genes for a specific metabolic pathway.

### **2.1.2. Introduction of Foreign Genes into Plant Cells**

Norman Borlaug, winner of the Nobel Prize in 1970, once said “Genetically modified organisms are the result of a natural process that was going on long before humans became involved”.

The foundations for recombinant DNA technology, based on genetic manipulation, were established when enzymes which modify DNA, such as endonucleases, ligases and polymerases, were discovered and were used by scientists to isolate, characterize and modify genes and ultimately transfer them to the same organism, or a different organism, to study and exploit their expression.

The development of transgenic plants involves the following steps:

- 1) Identification of a gene that could impart a useful character to the target crop plant and subsequent cloning of the gene. Strategies for this have been discussed above.
- 2) Modification of the target gene for expression in crop plants. The gene has to be isolated and cloned into a vector, which ensures the stable integration of the foreign gene into the chromosomes of the recipient plant cells. The gene must also be placed under the control of the appropriate regulatory sequences.
- 3) Transfer of the modified gene into cells of the plant species of interest by one of several methods, which will be discussed below.
- 4) Regeneration of complete transgenic plants capable of transmitting the incorporated gene to the next generation. This process involves the use of selectable markers, such as antibiotic resistance, in order to select for transformed tissue. The transformed nature of the tissue is confirmed by PCR, Southern and western blots or expression assays.
- 5) Phenotypic analysis of transgenic progeny to measure expression and functionality of the transgene in the transformed plants and segregation analysis of transformed progeny.
- 6) Field trials of transgenic plants.

Two classes of plant transformation technologies have been developed: *in vitro* methods, and “natural” methods. Among the *in vitro* technologies are microinjection

(not commonly used), direct DNA uptake into protoplasts (with or without electroporation), and microprojectile (or particle) bombardment. “Natural” technologies include the use of viral vectors (which lead to transient unstable transformation) and *Agrobacterium tumefaciens* mediated transformation. Each of these technologies has advantages and disadvantages. The *in vitro* techniques tend to result in transformed plants containing a high copy number of often rearranged or catenated transgenes, which can result in homology dependent suppression (transgene silencing). In addition, electroporation technology depends upon the ability to regenerate the transgenic protoplasts into whole plants, a process that is either difficult or impossible for many plant species. Particle bombardment can be performed with any tissue of most species; however, the process is relatively inefficient in that few cells are stably transformed. *Agrobacterium* transformation of most dicotyledonous species is simple and efficient, and methodology has been adapted to allow many monocotyledonous plants, including cereals, to be transformed. There remain, however, several important crop plants such as soybean and common bean for which efficient transformation systems are lacking.

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### Biographical Sketches

**Ana Tztzqui Chávez-Bárceñas** was born on July 1, 1970, at Uruapan, Michoacán, México. She finished her undergraduate studies in Biology from Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México. She undertook her M.Sc. studies in the Department of Plant Genetic Engineering in Centro de Investigación y Estudios Avanzados (CINVESTAV-I. P.N.), at Irapuato, Guanajuato, México, where she also completed her Ph.D. on Plant Biotechnology in CINVESTAV-I. P.N., Irapuato, Guanajuato, México. The investigation theme was on sucrose biosynthesis and carbon assimilation on rice, using transgenic plants as the model. She has published in *Plant Physiology*.

**María Fernanda Nieto-Jacobo** was born on March 12, 1973 in Guadalajara, Jalisco, México. She completed her undergraduate studies as a Biologist from Universidad de Guadalajara, Guadalajara, Jalisco, México. She completed her Ph.D. on Plant Biotechnology at CINVESTAV-I. P.N., Irapuato, Guanajuato, México. The investigation theme was on aluminum tolerance and mineral nutrition of sorghum transgenic plants over-expressing the citrate synthase gene from *Pseudomonas aeruginosa*. She has published on Plant Science, and in the Spanish-language version of *Scientific American*.

**June Kilpatrick Simpson Williamsom** was born on February 24, 1960 in Johnstone, Scotland, Great Britain. She completed her B.Sc. in Genetics from the University of Glasgow, Scotland, and her Ph.D. studies in Universiteit Gent, Belgium. She has been Professor in the Department of Plant Genetic Engineering in CINVESTAV-I. P.N., Irapuato, Guanajuato, México. Her main research interest is plant-pathogen interaction using the *Phaseolus vulgaris* - *Colletotricum lindemuthianum* system as the model. Research in her laboratory includes the use of traditional and molecular methods of analysis, and the use of molecular markers to map and study “Qualitative Trait Loci” (QTL’s) associated with drought tolerance in maize. She has published in *Nature*, *EMBO Journal*, *Science* and other international journals.

**Luis Rafael Herrera-Estrella** was born on June 21, 1956 in México, Distrito Federal, México. He completed his undergraduate studies in Biochemical Engineering from Instituto Politécnico Nacional, México. He completed his M.Sc. Studies in the Department of Genetic Engineering at the Centro de Investigación y Estudios Avanzados del I. P. N., México, D. F. He completed his Ph.D. and Postdoctoral Studies in Universiteit Gent, Belgium. He has been Professor at the Department of Plant Genetic Engineering in CINVESTAV-I. P.N., Irapuato, Gto. México, and Head of the *Biotechnology Education and Training Center* at the UNESCO. His main research interests are the development of genetic transformation methods to generate transgenic plants, the development of aluminum-tolerant transgenic plants and transgenic plants with improved nutrient uptake, the study of gene transcriptional regulation by light in plants, and the study of carbon assimilation and sucrose synthesis in plants. He has published in *Nature*, *Science*, *Cell*, *EMBO Journal* and others. Two of his papers in *Nature* are considered classical in molecular biology.