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Plant Biotechnology

Experience and Future Prospects

Second Edition

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Foreword

Reflections on Innovation and Progress

An examination of the meaning of words reveals that *innovation* and *progress* are strikingly inequivalent Examining their Latin derivations, *innovation* means entering into novelty by introducing something new into a preexisting device. *Progress* means going forward (*progressus*) and increasing (*progressio*). In the contemporary uses, *innovation* is a descriptive term, whereas *progress* includes an often positive value judgment. However, terminology may be a factor of progress or regression. Terminological conservatism (e.g., GMO) is an obstacle to scientific progress in some sectors. We cannot underestimate the power of words in social life as they condense real questions: How do we make scientific and technical innovation a progress for everybody? Is progress in science equivalent to progress in society?

The latter of these questions was addressed in a conference of the European Academy of Sciences in Brussels in 2016. The Standing Secretary of the French Academy of Sciences Catherine Bréchignac stressed that society embraces technology rather than science. A member of the French Academy of Technology, François Guinot, declared that technological innovation induces science to hide behind technology. Moreover, the exponential character of technological development creates a phase lag affecting many layers of society and resulting in political problems.

Innovation is a key factor of economic growth by producing new variations of preexisting devices, which provide consistency and the basis for innovation itself. Genome editing is a prominent example of true technological innovation based on a whole preexisting scientific story in fields such as bacterial genetics and virology. For example, the CRISPR-Cas9 system represents striking progress in genome cleavage thanks to its unprecedented precision. This system was significantly improved and simplified by Emmanuelle Charpentier and Jennifer Doudna awarded the Nobel Prize in Chemistry 2020.

Genome editing became a routine practice not only in biological and medical research, but also as a commonly used tool in agriculture and more applied in medicine. For example, success in the treatment of childhood leukemia was recently

reported. However, the European Union decided to consider genome editing as GMO technology. This label carried the consequence that it could not be used for agricultural production in Europe. Regarding medicine, eugenic practice on embryonic cells, which is different from editing somatic, differentiated cells in organs and tissues, was recently performed by a Chinese physician who was able to induce a gene mutation and prevent possible HIV infection in the baby. This practice was widely condemned. The French National Academy of Medicine and the French Academy of Sciences issued in 2018 a joint, balanced declaration, stating essentially: (1) in the present state of knowledge, it is not advisable to give birth to such embryonic-modified babies; (2) in case such a procedure could be started in the future, it must undergo academic and ethical approval and in-depth public debate; (3) responsible research using DNA-modifying technologies, including at the embryonic level, is important to human beings. Consequently, both Academies support such research.

At this point, we meet the question of the relationship between fundamental and applied research. Indeed, this relationship is bidirectional in its essence. "Translational medicine" consists of fostering applied, clinical testing in order to provide useful results for basic research. In this context, the question of risk evaluation arises, together with the famous aphorism "absence of evidence is not evidence of absence"—a quite ambiguous story with dubious logical foundations. Most of the time, the aphorism is used, according to the spirit of the *precautionary principle*, to prevent further research on supposedly toxic substances. Whether or not this use turns out to be justified, generally this aphorism should be used to foster research rather than to hinder it.

How can we ensure that scientific and technological innovation creates progress in society? Progress in society means applying several kinds of human values, not only knowledge, to the same effect. While progress is impossible without innovation, it is unimaginable if it does not pertain to society as a whole.

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The Tools for Engineering Plants

Chapter 1
The Evolution of Agriculture and Tools for Plant Innovation

Agnès Ricroch

Genetically engineered crops are playing an increasingly important role in world agriculture, enabling scientists to reach across genera for useful genes to enhance tolerance to drought, heat, cold, and waterlogging, all likely consequences of global warming. I believe biotechnology will be essential to meet future food, feed, fiber, and biofuel demand. The battle to ensure food security for hundreds of millions of miserably poor people is far from won. We must increase world food supplies but also recognize the links between population growth, food production, and environmental sustainability. Without a better balance, efforts to halt global poverty will grind to a halt.
Norman Borlaug—Science, 318: 359, 19 October 2007

Abstract Plants such as cereals and legumes on which humans depend on today were domesticated gradually and independently by ancient farmers in many different parts of the world over a few thousand years. Over time, ancient farmers converted hundreds wild species into cultivated crops (some of the world's most important crops). In the transition from foraging to farming 10,000 years ago, the wild forms of these plants mutated and were selected to result into new, domesticated species that were easier to harvest. This process continues today. Since the beginning of the twentieth century, innovation in plant genetic technologies has accelerated and produced better crops through increased resistance to pests and diseases, tolerance to drought and flooding, and biofortification. Together with the advancement of whole genome sequencing technologies dramatic and rapid progress has been made in our understanding and ability to alter gene expression in plants and in techniques for the identification, isolation and transfer of genes of interest. In many cases, this progress has been facilitated by the availability of efficient gene transfer methods, New breeding techniques (NBT) have rapidly emerged in the 2000s. Compared to the early versions of gene editing tools, such as ODM (oligonucleotide-directed mutagenesis), meganucleases (MNs), zinc fingers nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short

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palindromic repeat (CRISPR) system is capable of altering a genome more efficiently and with high accuracy. Most recently, new CRISPR systems, including base editors and prime editors, confer reduced off-target activity with improved DNA specificity and an expanded targeting scope. Geneticists use a wide variety of gene transfer methods to introduce foreign DNA (from microorganisms, plants, animals) into plants. Plant genetic improvement offer an effective approach to increase food production and food security in order to support the world's growing population, especially in inhospitable climates. Plant innovations can also improve production of medicines for all.

Keywords Griculture · Crop · Domestication · Breeding · NBT · CRISPR · Biotechnologies

1.1 Multiple Origins of Agriculture

Did you know that the oldest bee pollinators from 100 million-years ago were found in pieces of amber? Scientists also found evidence that human ancestors used fire one million years ago. According to fossils of starch grains from grinding stones and cooking pots found in archaeological sites, archaeologists stated that the history of plant breeding and cultivation of major cereals started about 10,000 years ago.

1.1.1 Emergence of Agriculture

The adaptation of crop plants to human needs and cultivation is a slow process evolving on a time scale of millennia. Wild cereals could have been cultivated for over one millennium before the emergence of domesticated landraces (Tanno and Willcox 2006). Human domestication of plants can be divided into three stages: "gathering," in which people gathered plants from wild stands; "cultivation," in which wild plants were systematically sown in fields of choice; and "domestication," in which mutant plants with desirable traits were raised (Weiss et al. 2006).

Based on recent DNA studies and radiocarbon dating of archaeobotanical remains, farming arose several times in several locations once the Ice Age had ended and climatic and environmental conditions were favourable for farming. Soon after humans adopted a sedentary existence agriculture arose (Tanno and Willcox 2006). These discoveries show the greatest revolution in human history: the transition from gathering foods from the wild to producing them on farms.

Foremost among the creations of ancient plant breeders are the cereals—rice, wheat, and maize, provide more than 50% of the calories consumed by humans today (Ross-Ibarra et al. 2007). However, 70% of the calories consumed by humans come from only 15 crops, which were domesticated in different countries worldwide. The Neolithic transition, which broadly describes the shift from foraging to farming, is one

of the most important events in human history. Agriculture happened first in the early villages of the Near East in the Fertile Crescent, a region from the Mediterranean Sea to Iran including modern-day Israel, Syria, Jordan northeastern Iraq and southeastern Turkey and subsequently occurred in different parts of the world including China, Mesoamerica and the Andes, Near Oceania, sub-Saharan Africa, and eastern North America (Riehl et al. 2013; Meyer and Purugganan 2013). As early as 13,000 years ago, hunter-gatherers first began to gather and plant seeds from wild cereals and legumes, such as wheat, barley, and lentils and began their cultivating more than 11,500 years ago. Plants were domesticated gradually and independently by people in many different parts of the world. Japonica rice, a subspecies of *Oryza sativa*, was bred about 10,000 years ago in the upstream region of the Yangtze River in China. Key crops such as rice and soybean originated in eastern Asia. This region is also the original home of several minor crops, such as certain types of millet. Maize eaten today by over 1 billion people was domesticated approximately 10,000 years ago in southwestern Mexico. For further information refer to the book «1491» by Charles C. Mann. Starting from 12,000 years ago in the Middle East, the Neolithic lifestyle spreads across Europe via separate continental and Mediterranean routes (Rivollat et al. 2020).

1.1.2 The 'Domestication Syndrome'

The dawn of agriculture, as well as of crop domestication, was a process of trials and errors. During domestication, humans subjected several key events to selection that make up the 'domestication syndrome'. During this process, ancient farmers, either consciously or unconsciously, saved seeds from plants with favoured characters to be sown the next year. The 'domestication syndrome' defined as phenotypic traits associated with the genetic change to a domesticated form of an organism from a wild progenitor form include loss of seed falling (shattering), decreased dispersal, loss of seed dormancy, increased number of seeds, change in seed shape, compact growth habit (reduced branching, reduced plant size, dwarfism), increased size of fruits, adaptation of flowering time to local areas, and reduced content of toxic compounds (safer food). Humans have also selected crops for disease-resistance.

The cereals—botanically a grass, from which the fruit which is called a caryopsis (grain) is harvested—, and most other crops, share a feature—a character or trait—central to domestication: their grains remain attached to the plant for harvest by humans rather than falling from the plant, as required by wild species to produce their next generation. For example, domestication of maize involved a plant architecture transformation from the wild ancestor (progenitor), *Zea mays* ssp. *parviglumis* resulting into an unbranched plant with seed attached to a cob, thereby making maize dependent on humans for cultivation. Subsequent to domestication, maize has been subject to intensive improvement efforts, culminating in the development of hybrid maize lines that are highly adapted to modern agricultural practices.Understanding the origins and domestication of crops is of evolutionary interest. Understanding crop

origins also allows the identification of useful genetic resources for crop improvement. Thus, domesticated plants provide a model system for studying adaptation of plants to their environment (the concept of adaptation is central in Darwin's work). Domestication shapes the genetic variation that is available to modern breeders as it influences diversity at the DNA level. Indeed, scientists today can follow how domestication proceeded at the level of DNA sequence change, from wild ancestors (progenitors) to cultivated crops. Insights into the domestication process reveal useful DNA information (at the gene level) for future crop breeding.

1.2 The Toolbox of Crop Improvement: Hybrids and First Biotechnologies

To accomplish the objectives for crop improvement plant breeders develop various tools and methods to broaden the possibilities for breeding new plant varieties: conventional breeding such as hybridization and mutation breeding, to advanced breeding techniques such as genetic modification.

The work of Charles Darwin (1859) and Georg Mendel (1866) created the scientific foundation for plant breeding (Fedoroff 2004). The Austrian monk Gregory Mendel showed the importance of statistics in breeding experiments and the predictability in selective breeding. In 1866 he formulated the laws of inheritance on garden peas and discovered of unit factors (later defined as genes). Previously, the French family of the Vilmorins, who established the first seed company in 1727 in France (today part of the Limagrain Cooperative), introduced the pedigree method of breeding in 1830 (based on selected individual plants). The first seed company in North America was established by David Landreth in 1784. He published a catalog of vegetable seeds in 1799. The twentieth century efforts were devoted to improving the productivity, reliability, and nutrition of crops: maize (George Beadle and Paul Mangelsdorf), fruits, vegetables, and ornamental flowers (Luther Burbank) to cite some. Indeed since the beginning of the 20th-century the plant breeder's toolbox has been developed to cause specific and permanent changes (genetic modifications): from first-generation hybrids (of maize and many other crops), wide-species crosses, mutation breeding, to genetic engineering. The new tools and methods are more and more rapid in their ability to create varieties with new and interesting traits.

1.2.1 Hybridization (Crosses Between Plants or Species)

The transfer of traits between genetically distant or closely related species is not a new technique. Hybridization which is a cross between two parental plants which carry interesting traits has been achieved in numerous crops. It takes almost 15–20 years to create a new hybrid variety such as in sunflower, maize, oilseed rape,

or wheat. In these wide crosses thousands of genes are affected while in transgenic plants one to six genes can be added (for the moment).

In 1919 in Connecticut Donald F. Jones developed the double-cross method in maize, which involved a cross between two single crosses (four inbred lines generated from the mating of parents who The domestication syndrome can be defined as the characteristic collection of phenotypic traits associated with the genetic change to a domesticated form of an organism from a wild progenitor form.are closely related genetically are used). This technique made the commercial production of hybrid maize seed economically-viable. In 1923 in Iowa, Henry C. Wallace developed the first commercial hybrid maize. In 1926, he then founded the Hi-Bred Maize Company (today Pioneer Hi-Bred, a DuPont Company).

Hybrid seed technology generates heterozygous plants with improved yield and disease resistance by adding traits from two different parents. Average maize yields over the past 40 years have doubled in the USA, but this did not occur everywhere in the world.

1.2.2 Chemical- and Radiation-Induced Mutagenesis

Chemical- and radiation-induced mutagenesis (using Gamma-rays and X-rays since ca.1920) increases the frequency of genetic variations which can be used to create new mutant varieties. A mutant is a plant/organism in which a base-pair sequence change occurs within the DNA of a gene or chromosome resulting in the creation of a new character or trait. These mutations can be interesting for crop improvement, such as reducing the height of the plant, changing seed colour, or providing tolerance or resistance to abiotic (e.g. salinity and drought) and biotic (e.g. pests and diseases) stresses. In the UK, much of the beer was produced using a mutant variety of barley (the 'Golden Promise' variety, salt-tolerant spring barley with semi-dwarfness in stature). Wheat varieties developed through mutation breeding technique are used today for bread and pasta (e.g. induced mutability for yield). Many physiological and morphological mutants have been obtained (in banana, cassava, cotton, date palm, grapefruit, pea, peanut, pear, peppermint, rice, sesame, sorghum, and sunflower … and also horticultural plants, see https://mvd.iaea.org/.) Over 3332 crop and legume varieties developed through chemical or radiation induced mutagenesis have been released worldwide in more in 73 countries: Seeds of tomato variety Bintomato-7 irradiated with gamma ray (370 Gy) were released in 2018 for cultivation in winter season (November-February) of Bangladesh, The mutant variety of wheat with low amylose was developed by treatment with chemical mutagen sodium azide (NaN3) in Japan; the first mutant semi-dwarf table rice 'Calrose 76' released in the US, a mutated indica rice stain developed by irradiation of seeds with gamma rays (250 Gy) with short stature (95 cm against 120 cm of Calrose), shortening of all internodes. In organic agriculture farmers use the 'Calrose 76' strain of brown rice, also developed through mutagenesis. Lewis J. Stadler of the University of Missouri was the first to use X-rays on barley seeds in 1920s and ultraviolet radiation on maize pollen in 1936.

Different kinds of mutagens are used in plant breeding, such as chemical mutagens like EMS (ethyl methanesulfonate) to generate mutants.

It takes more than ten years to create a variety with such mutations, which will be then crossed with an elite variety adapted to local agronomical and climatic conditions. Such varieties carry a huge number of genes affected. The random results of this genetic technique illustrate how spontaneous mutations create the genetic diversity that drives evolution (one of the Darwin's concept), and the material upon which selective breeding can operate.

1.2.3 Other Techniques: In Vitro Techniques, Genome Sequencing and Gene Mapping

Other breeding techniques using in vitro tissue culture—micropropagation, and embryo rescue—permits the crossing of incompatible plants and allows the production of uniform plants.

Thanks to the knowledge at molecular (DNA) level and bioinformatics the latest step of innovation in plant breeding, dating from the 1980s, came from biotechnologies. Molecular marker–assisted selection (MAS) is now widely used to localize characters or traits on the genetic map of the crop and select commercially important characters or traits. In MAS for example, a DNA marker closely linked to a disease resistance locus can be used to predict whether a plant is likely to be resistant to that disease (Tester and Langridge 2010).

In 1944, DNA as the genetic material was discovered in pseudococcus by Oswald Avery, Colin MacLeod, and Maclyn McCarty, from the Rockefeller Institute in the USA. Then in 1953 James Watson, Francis Crick, Rosalind Franklin, and Maurice Wilkins determined the structure of DNA. Since the 50s DNA sequencing has seen rapid progress. The first sequencing of a natural gene from yeast was made in 1965 and took 2.5 years. In 1976, the first genome was sequenced (a bacteriophage). In 2008, the first human genome (6 billion base pairs of DNA of James Watson's genome) was sequenced in four months and cost less than US$ 1.5 million. The price is dropping rapidly due to new DNA sequencing technologies. According to the National Human Genome Research Institute (USA) today the cost to generate a whole-exome sequence is generally below US $1,000.

A complete genome sequence is available for several crops since the late 1990s: bread wheat, rice, maize, papaya, grape, apple, soybean, potato, sorghum, strawberry, date palm, cassava, cacao, foxtail millet, cotton, banana… The latest sequenced genomes of 2013 are of chickpea, peach, sweet orange, and wild rice.

1.2.4 The Green Revolution

Since 1940, foundations such as the Ford, the Rockefeller, the Howard Buffet or the Bill and Melinda Gates Foundations have played a major role in collaboration with governments for breeding of crops. The Green Revolution started in 1943 when the Mexican government and the Rockefeller Foundation co-sponsored a project, the Mexican Agricultural Program, to increase food production in Mexico, in particular wheat production. Using a double-concept (interdisciplinary approach and international team effort), the scientific team headed by an American wheat breeder at the Rockefeller Foundation, Norman E. Borlaug, started to assemble genetic resources (germplasm) of wheat from all over the world. The life and legacy of the father of the Green Revolution, Borlaug, who received the Nobel Peace Prize in 1970, is celebrated in 2014 for the 100th anniversary of his birth.

After the famine of 1961 in India, Borlaug advanced the development of high-yielding varieties such as IR8—a semi-dwarf rice variety, along with expansion of irrigation infrastructure, and modernization of management techniques, distribution of hybrid seeds, fertilizers, and pesticides to farmers.

Today almost two billion people suffer from chronic hunger and malnutrition in developing countries. This makes agricultural development in developing countries a pressing need as they have the fastest population growth rate and they are also more at risk from resource shortages and the effects of climate change. Increasing food supply without deforestation or a net change in land use means increasing production. This makes agricultural development through crop improvement a pressing need. As deplored by Paarlberg (2009), modern agriculture—including biotechnology—has recently been kept out of Africa).

1.3 Advanced Breeding Techniques: Genetic Modification Technologies

In 1946 J. Lederberg and E. L. Tatum were the first to discover that DNA naturally transfers between organisms. Genetic engineering, also known as genetic modification (GM), exploits recombinant DNA technology as new tool for plant breeders. As a technique that is faster and able to deliver genetic changes that would never occur through conventional methods, GM is uniquely useful in the plant breeder's toolbox.

Conventional breeding today encompasses all plant breeding methods that do not fall under current regulations for GMOs. For example in Europe, the European legal framework defines GMOs and specifies various breeding techniques that are excluded from the GMO regulations (the European Directive 2001/18/EC on the deliberate release of GMOs into the environment). Excluded from this GMO Directive (and thus may be viewed as conventional breeding) are hybridization (cross breeding), in vitro fertilization, polyploidy induction, mutagenesis and fusion of protoplasts from sexually compatible plants. In the USA transgenic (GM) plants are deregulated

and not labeled as GMOs (except in some States). Edited plants are deregulated in the USA. The case of Europe is examined in the Joachim Schiemann's chapter.

1.3.1 Genetic Engineering Technologies

Transgenic techniques provide genetic modification or genetic engineering of a recipient plant with one or more foreign genes. These foreign genes can come from plant or non-plant organisms. Transgenic plants are used for precise crop improvement because of transfer of limited genetic material as oppose to conventional breeding in which one half of the genome from each parental line is combined after hybridization. Genetic engineering also makes possible genetic changes, including between animals and plants, which would be highly unlikely or would never occur using mutagenesis or other conventional breeding techniques.

Advances in molecular biology in the 1970s made it possible to identify the specific gene responsible for a trait, isolate it, and transfer it, from any type of organism, to plant cells. Instead of making tens of thousands of genetic changes (cross or mutation breeding), with transgenesis a gene with a known single beneficial trait is inserted into the plant genome. Plant breeders embraced transgenesis because it offered this precision and a quicker way of obtaining a desired trait in a plant.

Ethical questions on growing GM crops were addressed by scientists involved molecular biology research. The first GM experiment, published in 1972, described the insertion of bacteriophage genes into an animal viral DNA. Consequently scientists raised questions about potential risks of recombinant DNA to human health and organized the Asilomar Conference in 1975 in California in the USA, attended by scientists, lawyers and government officials to discuss the technology. They concluded that experiments could proceed under strict guidelines drawn up by the US National Institutes of Health (Berg et al. 1975).

There are several vectors to genetically engineer plants: (i) infecting plant tissue by recombinant *Agrobacterium tumefaciens* carrying a gene of interest will lead to integration of this gene in the plant DNA, a mechanism of genetic engineering discovered by Marc Van Montagu and Jeff Schell (in Belgium) and Mary-Dell Chilton (in the USA) in 1977, or (ii) shooting plant tissue with a 'particle gun' carrying tungsten or gold particles coated with the gene to be transfered (also called as biolistic particle delivery system; developed in 1984 by John Sanford, Edward Wolf, and Nelson Allen in the USA).

Introduced genes fall randomly amid the DNA strands. Plant mutation breeding (discussed above, 2.2) may induce more changes than transgene insertions through genetic engineering. Regeneration of a genetically engineered plant is a rather fast process, however, since such a variety need to be crossed with elite varieties adapted to specific agronomical and climatic conditions, it takes a few years to create a variety with added transgenes.

A special feature of genetic modification is that it allows the transfer into crop plants of one or a few genes from unrelated organisms (microorganisms such as

bacteria, animal or human). Conventional breeding (hybridization between very distinct plants even from different genus) cannot form plants with genes coming from different kingdoms. Additional techniques of modern plant breeding are discussed in the second chapter by Surinder Chopra.

1.3.2 Traits Expressed by the Genetic Engineering Technologies

The first GM plant produced was an antibiotic-resistant tobacco plant in 1982. The first commercialized GM crop was the FlavrSavr® tomato in 1994 in the USA. It contained a trait that suppressed early ripening in tomato to maintain flavor and taste. In the UK, a concentrated tomato paste using these GM tomatoes went on sale in 1996 (by Zeneca). It received an award in France for the best innovation. The earliest crops produced by transgenesis (insect-resistant and herbicide-tolerant varieties) have been commercially cultivated since 1995. A GM variety of maize developed to express a protein from *Bacillus thuringiensis,* (‘*Bt* maize’) protects maize against the European maize borer and some other lepidopteran insects. *Bt,* originally discovered in 1911 in the province of Thuringia in Germany, has been used as a spray by organic farmers. The *Bt* genes produce insecticidal CRY proteins which are an alternative to chemical pesticides. These are introduced in more than a thousand elite varieties of maize, but also in cotton, cowpea, soybean and sugarcane as examples.

The global area cultivated with GM varieties was over 191.7 million hectares in twenty-six countries (21 developing and 5 industrialized countries) in 2018. A total of 26 countries adopted GM crops through cultivation and 44 additional countries imported. Crops grown commercially today contain traits for mainly herbicide tolerance, insect resistance, or both. These have been developed for commodity crops such as soybean, cotton, maize, oilseed rape and alfafa. It is estimated that, for example, 88% of the cotton grown in India is now GM due to its greater resistance to pests. The cultivation of GM insect-resistant crops, particularly varieties of cotton, in India and China, is also reducing the exposure of farmers to harmful organo-phosphate insecticides. There are a lot of products from GM crops in the food chain. In Europe it is estimated that 90% of some animal feed (maize and soybean) is derived from GM varieties because of their low cost and large amount available.

The list of approved GM crop varieties modified by transgenesis (gene transfer or silencing using RNAi) is long: alfalfa, Argentine canola, apple, bean, canola, carnation, creeping bentgrass, cotton, cowpea, eucalyptus, flax, maize, melon, miscanthus, papaya, petunia, plum, Polish canola, potato, rice, rose, squash, safflower, sorghum, sugar beet, sugarcane, sweet pepper, soybean, tobacco, tomato, wheat (for updated data visit https://www.isaaa.org/gmapprovaldatabase/default.asp). The list of edited crop varieties which are deregulated includes e.g. alfafa, bahiagrass, camelina, citrus, chrysanthemum, flax, maize, pennycress, Petunia, potato, rice, setaria (wild millet), soybean, tobacco, tomato or wheat.

Many genes of interest have been discovered including pest and disease (fungi, virus, bacterial) resistance genes, and new ones are being discovered at a rapid rate. Some of these genes have been incorporated into commercial varieties to breed for specialty traits and these include heat and drought tolerance, nitrogen use efficiency, modified alpha amylase, male sterility, modified amino acid, modified flower color (in dianthus), modified oil/fatty acid, and virus resistance. In Pamela Ronald's laboratory in UC Davis (USA) the discovery of the gene XA21 confers resistance to a bacterial disease, and the discovery of a gene of submergence tolerance of rice allows drowning weeds without drowning the rice, providing a method for weed management without relying on a herbicide (Ronald and Adamchak 2008).

Radical innovations concern nutritional benefits. Healthier vegetable oils with fewer trans-fats are being developed. Bio-fortifying key crops including cassava in Africa or rice in Asia illustrate the potential of genetic engineering to fight malnutrition. In developing countries, especially in Asia, vitamin-A deficiency causes childhood blindness. The most famous attempt to combat this deficiency is the development of 'Golden rice' by Ingo Potrykus in Switzerland and his colleagues (Zeigler 2014). They genetically transformed rice plants with carotenoid biosynthetic genes that result in more vitamin-A precursors. Today, geneticists are also trying to reduce allergens in foods using genetic engineering. Technologies such as genomic selection, genome editing and the role of bioinformatics could be galvanized by using speed breeding to enable plant breeders to keep pace with a changing climate and environment in plant adaptation to environmental and biotic contraints,

The ability to manipulate plant genes to produce certain human enzymes is not new. Interest in deriving pharmaceuticals from plants (known as 'bio-pharming'), first took off in the 1990s after scientists showed that monoclonal antibodies could be produced in tobacco plants. Plant-derived biologic treatments have proven successful in drugs given to animals in recent years and today in human patients suffering from Gaucher disease or development of vaccines against COVID-19 (discussed in Kathleen Hefferson's chapter). This led to genetic engineering of plants to produce vaccines, antibodies and proteins for therapeutics.

1.3.3 Development of New Breeding Techniques

In the past two decades, additional applications of biotech and molecular biology in plants have emerged, with the potential to further enlarge the plant breeder's toolbox. Making precise changes in the genomes of organisms is challenging for most techniques. Several recently described genome editing techniques allow for site-directed mutagenesis of plant genes (to knock out or modify gene functions) and the targeted deletion or insertion of genes into plant genomes. New breeding techniques have rapidly emerged in the 2000s. Compared to the early versions of gene editing tools, such as ODM (oligonucleotide directed mutagenesis), meganucleases (MNs), zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeat (CRISPR)

system is capable of altering a genome more efficiently and with high accuracy. In 2012, researchers transformed a bacterial immune system (CRISPR system) into a fast and versatile tool for genome editing. The Royal Swedish Academy of Sciences has decided to award the Nobel Prize in Chemistry 2020 to Emmanuelle Charpentier (Max Planck Unit for the Science of Pathogens, Berlin, Germany) and Jennifer A. Doudna (University of California, Berkeley, USA) for the development of a method for genome editing. Since 2014 plants were edited with CRISPR-cas9 and notably the hexaploid wheat (Ricroch 2017). Regarding plant species and countries in which the research is performed, one can note the importance of rice, mainly in China, which is in accordance with the Chinese research and economic contexts, while the application of CRISPR/Cas systems in maize is more prevalently studied in the USA (Ricroch et al. 2017). China is now taking the lead in the industrial and agricultural applied sectors and in the total number of patents per year (Martin-Laffon et al. 2019). Another innovative trend is the use of transgenes solely as a tool to facilitate the breeding process. In this application, transgenes are used in intermediate breeding steps and then removed during subsequent crosses, eliminating them from the final commercial variety (null segregants). Among new tools are accelerated breeding techniques, where genes that promote early flowering are used to speed up breeding, and reverse breeding, a technique that produces homozygous parental lines from heterozygous elite plants (Lusser et al. 2012). New tools also concern three techniques: cisgenesis, intragenesis, and the zinc finger nuclease-3 technique (ZFN-3). Cisgenesis is the genetic modification of a recipient organism with a gene from a crossable–sexually compatible organism (same species or closely related species). Intragenesis is a genetic modification of a recipient organism that leads to a combination of different gene fragments from donor organism(s) of the same or a sexually compatible species as the recipient. ZFN-3 allows the integration of gene(s) in a predefined insertion site in the genome of the recipient species. In 2012, the researchers transformed a bacterial immune system into the fast and versatile tool for genome editing (CRISPR system).

A search-and-replace method, also known as prime editing, was developed that can introduce user-defined sequence into a target site without requiring double-stranded breaks (DSBs) or repair templates (Anzalone et al. 2019). For precision breeding of crops this genome engineering using prime editing system was developed in rice (Hua et al. 2020) and wheat (Lin et al. 2020). China and the USA lead scientific research in crop editing while Nigeria being headquarters to numerous research consortia mainly using transgenesis (Ricroch 2019).

1.4 How to Meet 70% More Food by 2050?

Global population has risen from 2.6 billion in 1950 to around 7.8 billion in 2020, and is predicted to rise to a world population of near 10 billion people by 2050. According to the Food and Agricultural Organization of the United Nations, the demand for food could rise by 70% by 2050. To meet this goal an average annual

increase in production of 44 million metric tons per year is required, representing a 38% increase over historical increases in production, to be sustained for 40 years.

This accomplishment will be particularly challenging in the face of global environmental change. The challenge for major changes in the global food system is that agriculture must meet the double challenge of feeding a growing population, with rising demand for meat and high-calorie diets, while simultaneously minimizing its global environmental impacts (Seufert et al. 2012).

Today farmers will have to hit targets for reducing greenhouse gas emissions, improving water use efficiency and meeting the demands of consumers for healthful food and high-value ingredients. In this context, new plant breeding techniques are needed to contribute to improvements in crop productivity and sustainability in a climate-smart agriculture framework.

New technologies must be developed to accelerate breeding through improved DNA methods and by increasing the available genetic diversity in breeding germplasm (collection of wild types and varieties). Scientists underline the importance of conserving and exploring traditional germplasm. Introgression of characters or traits (pest and disease resistances or adaptation to salinity, cold or heat temperatures for example) into locally adapted varieties is expected to considerably enhance productivity in protecting crops from new pests and diseases due to climate change variability and under abiotic stress conditions (e.g. drought). The most gain will come from delivering these technologies in developing countries, but the technologies will have to be economically accessible and readily disseminated.

With governments, the private sector, foundations, and development agencies faced with feeding a growing and hungry world, research to increase agricultural productivity and access to affordable and safe medicines is needed including against COVID-19. The rush to develop a vaccine for COVID-19 the disease caused by the novel coronavirus SARS-COV-2 has extended to public and private laboratories, where scientists are using the tools of genetic engineering to develop edible vaccines in plants. The challenges of intellectual property rights and genetic resources preservation that play major roles in the plant breeding enterprise. The twenty-first century will witness radical plant innovations.

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Chapter 2
Techniques and Tools of Modern Plant Breeding

Dinakaran Elango, Germán Sandoya, and Surinder Chopra

Abstract Field and vegetable crops are primary source of food. Field crops are also a rich source of cellulosic biomass and carbohydrates for biofuels. One of the major challenges facing agriculture today is improving the productivity of crops in an environmentally sustainable manner. Annual climate variation causes temperature extremes, floods, and droughts which all exacerbate the vulnerability of crops to pests and diseases. Conventional plant breeding has evolved and molecular and modern breeding methods have enhanced the pace of crop improvement work. Plant breeders now use molecular and genetic techniques to selectively identify phenotypes and genotypes that are associated with traits of interest. Such functional genomics studies help plant breeders efficiently utilize the germplasm. Cutting edge molecular tools are now available in economically important crops as well as model plant systems. Gene expression techniques have been combined with forward and reverse genetic methods for isolation and introgression of desirable alleles into breeding populations that are used to develop hybrid crops. This chapter focuses on modern techniques and resources that field and vegetable crop scientists use to generate genetic information and efficient breeding strategies.

Keywords Association mapping · Genetics · Genomics · Germplasm · Marker assisted selection

2.1 Plant Breeding and Plant Ideotypes

Plants are the primary source of food, feed and energy and without them life on earth cannot be imagined. With a tremendous increase in human population, dramatic variability in the climatic patterns from year to year-enhanced efforts are needed to breed efficient plants. Plant breeding specifies plant improvement which can be attained

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either via sexual or asexual methods of propagation. Plant breeding process may involve domestication of a plant species from its wild native environment, developing pure lines, single or double cross breeding, and finally developing hybrids. The science of plant breeding relies on the principles of genetics, information on chemistry and physiology of metabolic pathways, and growth and development of the plant. On the other hand, plant breeder has a special art or a skill and an eye for selecting plants with morphological traits (phenotypes) or features that conform to a preconceived ideotype. A plant breeder also focuses on development of pest, disease and stress tolerant varieties. Thus, depending upon the plant organ to be harvested and climatic conditions for growing that particular variety, the definition of an ideotype can be developed. Ideotype development is dictated by how efficiently a plant utilizes natural resources. Modern plant breeders have several genetic tools and techniques available, which can be used to enhance the process of final product development.

2.2 Plant Breeding Exploits Phenotype and Genotype

Phenotype is the result of interaction of genes of a plant among themselves and with the environment in which the plant is growing. The biological processes involved in plant growth and development are complex and influenced by individual genes as well as a combination of several genes. Traits that are controlled by single genes give rise to qualitative variation while multigenic traits produce quantitative variation. Such quantitative traits exhibit complexity and are highly influenced by environmental conditions. Plant domestication is one of the examples of phenotypic selection in which by growing a wild form the ancient farmers have selected modern types. This is best exemplified by the domestication of teosinte into our modern maize. One can find landraces of field crops as well as horticultural and ornamental plants, which represent selections made by farmers and breeders in a specific climatic condition or in a geographical region. A plant breeder can now make use of sophisticated phenotyping tools to precisely measure the phenotypic effect of a trait.

Mendel's laws provided the genetic basis of segregation of traits (genes) and as the science of plant breeding evolved, a successful program combines traits from different germplasm sources by hybridizations (crossing). After hybridization a plant breeder then grows the subsequent generations to select the best combinations. Cultivars were developed by the use of breeding methods like pure line selection in a self-pollinated crop like wheat. One drawback of pure line breeding has been the genetic homogeneity, which caused instability especially during the growing season when a new race of a disease appeared. In open pollinated crops however, random mating of plants within a population followed by selection provided some advantage by selecting a population that performed better. Thus, phenotypic selection used by conventional plant breeders and success of this art of selection has been well documented in the form of release of high yielding inbreds, hybrids and varieties.

2.3 Molecular Markers and Plant Breeding

In traditional plant breeding, genetic composition of the populations and progenies was not known. However, with the availability of DNA sequences for several plant genomes it is now possible to develop molecular markers. Examples of commonly used include SSR (Simple Sequence Repeat) also known as satellite markers, and SNP (Single Nucleotide Polymorphic) markers. These reliable markers are based on PCR (Polymerase Chain Reaction) methodology. First and foremost, markers are used to enhance the process of breeding and this method is called marker assisted selection (MAS) based on the polymorphisms between the two parental lines used in a cross. The segregating progenies from F_2 (second filial) generation onwards are then screened using the markers that are genetically linked with specific traits in one or the other parental line. Plant breeders also use molecular markers in mapping of genes. In rice, for example, the *Sub1* locus which provides tolerance to submergence, was introgressed from a landrace of *Oryza sativa* into rice cultivars by the use of a method known as marker-assisted backcrossing (MAB) (Septiningsih et al. 2009). In lettuce, several single genes were mapped for disease resistance and MAS can be developed but pathogens are in constant evolution making these markers ineffective. By the use marker assisted breeding, genes for salinity tolerance have been introgressed in wheat and rice. Simple traits that are controlled by single genes can be mapped with relative ease by the use of molecular markers through backcross breeding. DNA-gel-blot-based restriction fragment length polymorphic (RFLP) markers have been used previously in maize, sorghum, and barley to identify quantitative trait loci (QTL) of complex traits conferring tolerance to drought and diseases. In addition to gene and QTL mapping, molecular markers are used in association mapping studies at the single candidate gene level. There are several examples of association of candidate gene and QTL with a given trait. In maize, genome wide association studies (GWAS) allowed the identification of loci associated with leaf length, width and angle. Flowering time variation analysis in maize has led to the association of markers in *dwarf8* gene, provitamin A and molecular markers in the *IcyE* gene in maize. Several agronomic traits in rice have been associated with markers based on single nucleotide polymorphism (SNP). The same applies to vegetable like lettuce with less studied cases; the significant relationship between a marker and a trait was identified for shelf-life (Kandel et al. 2020).

Technological innovations have led to modern genotyping platforms that evolved from laborious gel-based methods. These innovations have also reduced the cost of DNA sequencing which in turn has improved the efficiency of generating new markers to assist with MAS. These genotyping methods have been employed for field crops like rice, maize, barley, and wheat. The re-sequencing efforts of diverse rice germplasm through the Rice SNP Consortium (https://www.ricesnp.org) have provided valuable information on millions of SNP markers. Vegetable crop species as lettuce with larger genomes have been sequenced within the *Compositae* family, one of the largest in the plant kingdom.

2.4 Recombinant Inbred Lines for Plant Breeding

Plant breeders rely on natural variability in order to exploit genetic diversity available within a plant species. Molecular markers that are associated with specific traits are then used to identify diverse germplasm of economically important crops like maize, wheat, sorghum and soybeans. Plant scientists have developed resources to tap genetic diversity and these are used for GWA mapping studies. For example, in maize, nested association mapping (NAM) RIL (Recombinant Inbred Line) populations have been developed (https://www.panzea.org) by crossing twenty-five diverse parental lines with B73, a common parental line (200 RILs per cross). These 5000 RILs capture approximately 136,000 recombination events. These and other plant breeding resources that capture natural variation or genetic diversity allow plant breeders to study the effect of different alleles that are present in diverse parents used for developing association mapping panels. In self-pollinating species populations such as Multi-parent Advanced Generation Inter-Cross (MAGIC) are developed by intercrossing multiple diverse parents to generate a mapping population with a wider genetic background to detect QTLs (Huang et al. 2015). Breeding lines can be also derived from individual families used for mapping.

2.5 Plant Breeding with Haploids

Haploid breeding allows to achieve homozygous condition and this property is very crucial for quick release and dissemination of plant cultivars (Dwivedi et al. 2015; Gilles et al. 2017). Haploids were first reported in Jimson weed (Blakeslee et al. 1922), and later in several crop species. The commercial exploitation of haploids in plant breeding was recognized only after the discovery of anther-culture in Datura. There are numerous ways of generating haploids in plants. One of the recent approach is CENH3 mediated chromosome elimination to generate haploids (Ravi and Chan 2010). Other notable methods to create haploids are anther culture, interspecific and intergenic hybridizations, agrobacterium-mediated transformations, and haploid inducer lines. Heterosis was realized in maize after the discovery of haploid inducer lines. Later the gene underpinning the genetic regulation of haploid induction was identified as NOT LIKE DAD (NLD)/MATRILINEAL (MTL)/ZmPHOSPHOLIPASE A1 (ZmPLA1) in maize.

2.6 Speed Breeding

One of the key bottlenecks for plant breeding is the long generation times of crops, which hinders the rapid development of new crop varieties. Scientists at the University of Queensland during 2003 coined the term ‘speed breeding’ for set of improved

methods to hasten wheat breeding program (Hickey et al. 2019). Speed breeding reduces the crop cycle by extending the photoperiods using artificial lights with controlled temperatures in a growth chamber (Hickey et al. 2019). Speed breeding protocols were developed for important field crops and are being developed for other orphan as well as short-day crops like sorghum. Speed breeding accelerates the rate of genetic gain which helps in fast forward genomic selection, express genome editing. This technology can be adapted according to the crop needs as some of the plants are sensitive to constant light.

2.7 Genome Wide Association Mapping in Plants

GWA mapping studies have been extensively conducted to dissect the genetic causes of complex traits. GWAS is powerful because of high allelic diversity, recombination rates, and the availability of molecular markers densely distributed across genomes. Advent of high throughput genome sequencing technologies made it possible to sequence large number of germplasm collections with an affordable price. The rare alleles present in the wide collection of germplasm materials could be tapped in plant breeding using GWAS. Core, mini-core and association mapping panels have been developed for different field crops and successfully utilized in GWAS to identify promising candidate genes and QTL regions. Novel traits like epi-cuticular wax genes were mapped using such panels in sorghum (Elango et al. 2020).

2.8 Availability of Sequenced Genomes of Field Crops

With the advent of modern DNA sequencing technologies, several plant genomes have been sequenced and are publicly available (https://www.gramene.org/info/about/species.html). These genome sequences provide tremendous opportunities of efficient crop improvement. First of all, genome sequences are rich sources for developing molecular markers. As explained above, these markers can exploit polymorphisms among germplasm lines of that plant species. Plant breeders can then perform allele mining based on these sequence polymorphisms and use selected alleles in the breeding program. Secondly, plant breeders use these reference genome sequences to perform gene mapping of the traits of interest.

2.9 Plant Breeding and Gene Expression Techniques

Crick (1970) described the Central Dogma of molecular biology in which the genetic information from DNA is converted first into RNA, which is then translated into protein. Over the past four decades, the science of molecular biology has exploded

because of innovations in technology as well as computational biology. Current focus of a crop improvement program is to develop strategies and decisions based on gene expression. These expression-based techniques help identify, validate, and use desirable genes in the breeding programs. Field crop scientists are now routinely using gene expression as a molecular marker to decide about the strength of an allele of the given gene. Gene expression technologies include expressed sequence tags (ESTs), which are short cDNA (complementary DNA) sequences that can provide information about the expression of genes. EST sequences available for different plant tissues can provide tissue-specific or tissue-preferred expression data. EST sequences are now being used to develop gene-specific markers of expressed genes that crop scientists use in MAS breeding projects. DNA microarray is a gene expression technique in which DNA of all the genes of a plant species is fixed on a slide or a support. These slides are then used to hybridize with RNA from the same tissue of different parental lines or different tissue of the same parental line. DNA microarrays thus provide RNA expression information (i.e. similarities and differences) among different breeding lines as well as tissue-specific changes of genes. RNA-seq is another gene expression analysis tool which generates large data sets from a high throughput sequencing platform. Bioinformatic techniques have been developed to statistically analyze large gene expression data sets. RNA-seq thus provides global gene expression from thousands of genes and this analysis can be extended to multiple breeding lines. The Illumina based sequencing platforms HiSeq and MiSeq can be used for multiplexing large number of samples and these innovations provide huge data on expression of thousands of gene for hundreds of parental lines. These high throughput sequencing (HTPS) techniques have provided gene expression data for important field crops and vegetables. Gene expression profiling has further revolutionized the characterization of complex traits, which are controlled by multiple genes and their effects have been mapped as QTL. The association between phenotype and genotype by the use of molecular markers is done during the identification of a QTL. Expression QTL (eQTL) utilizes the concept of traditional QTL mapping in concert with genotyping information from transcription profiling data. Agronomically important traits are complex traits and eQTL mapping offers an efficient breeding tool. These marker–trait associations have been further exploited by validating them in order to use the relationship across different related species. For example, a major QTL identified in maize has been employed in sorghum to achieve virus and downy mildew resistance. More than 5,000 eQTLs regulate the expression of 4,105 genes; of which 9 eQTLs associated with flavonoid biosynthesis in addition of 6 loci likely responsible for anthocyanin variation in lettuce leaves which gives the red characteristic to leafy lettuce.

2.10 Forward Genetics for Plant Breeding

The goal of forward genetics is to identify the genetic variation underlying a trait. Mutants or variants are either naturally existing or new mutants can be generated artificially. New natural mutations occur at low frequency because these are the direct result of the evolutionary processes. Naturally occurring mutations represent the types that have adapted to a certain environment or a disease or insect pressure. Since naturally occurring mutations are not found for all traits, especially for traits of agronomic importance, plant breeders use artificial methods of generating mutations. Mutation breeding involves use of chemical, physical and insertional mutagens to generate new mutations and then identify plant phenotypes. Commonly used chemical mutagens are methyl nitroso urea (MNU) and ethyl methane sulfonate (EMS). EMS causes single base pair changes and can produce large number of mutations per kilobase pair of the DNA. Dominant mutations are screened in the M_0 generation. However, selfed progeny of the M_0 plants give rise to M_1 where segregation of traits takes place and thus recessive mutations are then identified in the M_1 generation. Physical mutagens including gamma rays and fast neutrons have been used to generate deletions in chromosomes. In the forward breeding programs, mutations are selected and introgressed to improve crops. A highly efficient EMS mutant library was developed recently in sorghum. EMS has been used to mutate vegetable populations of lettuce for seed germination at high temperatures resulting in families with higher germination at high temperature and for tolerance to herbicides (Huo et al. 2016).

The third types of mutations are caused by insertion or transposable elements or jumping genes. Barbara McClintock was awarded a Nobel Prize for her discovery of transposable elements in maize. Transposable elements excise from one place in the genome and re-insert at another place randomly. When transposons jump into a region of the gene that encodes a protein, the function of that gene is disrupted giving rise to a mutation. Today, plant scientists use transposable elements as genetic tags that can be used to clone the flanking gene sequence for which the mutation resides. Similar to the transposons, T-DNA (transfer DNA) has been used to develop insertion libraries of mutants. T-DNA is a plasmid DNA present in the engineered *Agrobacterium tumefaciens* and which can be engineered to transfer genes of interest. T-DNA has been used as an insertion tool to carry certain marker and/or antibiotic or herbicide resistant genes into plant cell. Public resources of maize, sorghum, brachypodium and rice are available for crop scientists to screen their desired mutations. Once a mutation is identified, a co-segregation analysis is performed in segregating generations to associate the insertion allele with the novel phenotype. In Arabidopsis as well as in many of the vegetables to date sequenced genomes have been valuable source of information for researchers to screen for mutations.

In forward genetics, once a mutation is identified, a mapping population is developed by crossing the mutant line with another parental line with large number of polymorphisms. In addition, the second parental line is chosen based on the availability of other genetic and genomic resources developed from this particular parental

line. For example, the maize inbred line B73 has been used to develop the reference genome sequence, transposon insertion databases, and availability of transcriptome, proteome and metabolome resources and databases.

2.11 Reverse Genetics Tools

Plant breeding efforts have been enhanced by the availability of genome sequences. One of the challenges is to ascribe a function to a putative gene sequence. Forward genetics can identify a limited number of phenotypic mutations followed by the mapping of the underlying genes. Reverse genetics utilizes the available genic sequence to identify its function by developing gain of function or loss of function mutants. Reverse genetics tools thus allow crop scientists to dissect the function of a putative gene sequence. Transposon and T-DNA insertion libraries are extensively used for reverse genetics in Arabidopsis. Several functional genomics techniques have been made available to perform reverse genetics in crop plants including maize, sorghum, rice and tomato (Char et al. 2020; Emmanuel and Levy 2002; Ram et al. 2019).

Chemical mutagenesis via EMS has been advanced to the isolation of mutants by the use of TILLING (Targeting Induced Local Lesions in Genomes; Till et al. 2006). TILLING has been successfully used for maize, rice, wheat, barley, and sorghum and vegetables as lettuce. Insertional mutagenesis using transposons and T-DNA transgenes has been one of the popular reverse genetics' techniques. Insertion elements are dispersed throughout the genome and such plant populations are then used to screen presence of insertion in the gene of interest. In general, insertion mutagenesis identifies loss of function mutations. However, there are examples in maize where 'gain of function' mutations have been identified as well.

RNA induced gene-silencing method also known as RNA interference (RNAi) is being used to study the function of a known sequence. In RNAi mutagenesis, plants are transformed with a vector which generates a double stranded RNA corresponding to the gene of interest. Synthesis of double stranded RNA in the plant cell triggers the cellular machinery to degrade the RNA produced from the gene of interest. Similar to RNAi, virus induced gene silencing (VIGS) has been used as a reverse genetics tool; but compared to RNAi, VIGS is relatively quick because it does not involve development of transgenic plants. Thus, VIGS produces transient phenotypes that are not heritable, while RNAi generated mutations are heritable and can be deleterious. The advantage of RNAi over KO (knock out) insertions is that if the KO mutation is lethal, RNAi will usually be not lethal and thus allow isolation of mutants with reduced expression level.