Chapter 13
Mutation Breeding and Drought Stress Tolerance in Plants

Mohammad Taher Hallajian

13.1 Introduction

Mutagenesis is a process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation. It may occur spontaneously in nature, or as a result of exposure to mutagens. Mutagenesis in the laboratory is an important technique whereby DNA mutations are deliberately engineered to produce mutant genes, proteins, strains of bacteria, or other genetically modified organisms. Various constituents of a gene, such as its control elements and its gene product, may be mutated so that the function of a gene or protein can be examined in detail. The mutation may also produce mutant proteins with interesting properties, or enhanced or novel functions that may be of commercial use. Plant mutagenesis is rapidly coming of age in the aftermath of recent developments in high-resolution molecular and biochemical techniques. By combining the high variation of mutagenized populations with novel screening methods, traits that are almost impossible to identify by conventional breeding are now being developed and characterized at the molecular level [1]. Mutation induction continues to contribute to crop improvement, using physical mutagens such as gamma ray, X-ray, fast neutron, and chemical mutagens such as ethyl-methane-sulfonate (EMS) and sodium azide (IAEA 2009). Ionizing radiation includes ultraviolet (UV) light, X-ray, gamma rays, and neutrons. These high-energy forms of radiation cause double-strand breaks of the DNA double helix. Radiation causes deletions of nucleotides from the DNA sequence. These deletions can cause reading-frame

M.T. Hallajian (✉)
Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute, Karaj, Iran
e-mail: mhallajian@nrcam.org

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shifts, inactive protein products, or faulty transcripts. Chemical mutagens affect the DNA molecule through chemical reactions within the genome. Base analogues are chemicals with similar properties to the DNA bases. They can be incorporated by the cell into the genome, replacing the proper base such as alkylating agents, nitrous acid, and intercalating agents [2]. Transposable elements are a special class of mutagen. They are self-replicating segments of DNA that excise and/or insert themselves within the genome. Also known as transposons, these strange sequences were first proposed by the pioneering Barbara McClintock working on maize [3]. Transposable elements, unlike other forms of mutagenesis, do not act upon the genome in a completely random fashion [4].

Among physical mutagens, gamma radiation has been widely used for mutation induction of both seed and vegetative propagated crops. Recently ion energy technology—heavy ion beam (HIB) and low energy ion beam (LIB)—is being utilized for mutation induction in wide-ranging crops. HIB is predominantly used for inducing mutations in plants. The spontaneous mutation rate is pretty low and cannot be exploited for breeding and that is why artificial mutations are induced with physical and chemical mutagen treatment. Many useful genetic changes have been induced by mutagen treatment including high yield, flower color, disease resistance, early maturation, and so on in crops, vegetables, medicinal herbs, fruit, and ornamental plants. However, the major problem with fruit-breeding work is the long life cycle of many fruit crops, which varies from 3 to 25 years or even more. In fruit crops, mutagenesis has been quite useful in isolation of useful mutants such as plant size, blooming time, fruit ripening, fruit color, and resistance to pathogens [5].

13.2 History of Mutation Breeding

Historically the use of mutagenesis inbreeding has involved forward genetic screens and the selection of individual mutants with improved traits and their incorporation into breeding programs. DeVries [6] suggested the use of radiation to induce mutations. The discovery that X-ray induced mutations in *Drosophila melanogaster* [7] and *Hordeum vulgare* [8] led to the use of radiation-induced mutations for changing plant traits by plant breeders and geneticists [9]. Since the discovery of X-rays about 100 years ago, the use of ionizing radiation, such as X-rays, gamma rays, and neutrons for inducing variation, has become an established technology [10]. Mutagenesis has been used in plant breeding since Muller's discovery of the mutagenic effects of X-rays on Drosophila flies [7]. Induced mutations have been used in the improvement of major crops such as wheat, rice, barley, cotton, peanuts, and beans, which are seed propagated [10].

Mutation breeding became more widely used in the 1950s, after the US atomic bombing of Japan at the end of World War II in 1945. In the wake of the

devastation, there was a desire to find uses for the "peaceful atom" that were helpful to humanity. Atomic gardens were set up in the United States and Europe, and even in Japan, with the aim of creating high-yielding and disease-resistant crops.

Hermann J. Muller, founder of mutation genetics and winner of the Nobel Prize in Physiology or Medicine in 1946, summed up the broad range of aspects and implications of mutation research in his Nobel Lecture on "The Production of Mutations."

Auerbach and Robson (1946) reported the use of chemicals such as mustard gas to be highly mutagenic. Since then a number of agents have been discovered that can increase the frequency of artificially induced mutations [9].

Atomic gardens, built around gamma-ray emitters, were popular among breeders in the 1960s and Japan still operates one. China began launching seeds into space in 1987 to take advantage of cosmic radiation and low gravity, developing more than 40 mutant crops with higher yields and better disease resistance, including varieties of rice, wheat, and pepper [11].

Bhabha Atomic Research Centre (BARC) developed 41 new crop varieties (Trombay varieties) by radiation-induced mutation and crossbreeding; these have been released and officially notified by the Ministry of Agriculture, Government of India for commercial cultivation. It started in 1973 with Trombay Groundnut (TG-1) cultivated mainly in Gujarat and Maharashtra [12].

Ion beams have been widely used in the research on material surface modification since the 1970s. Their application for mutation induction was started with low-energy ions in China in the late 1980s and with heavy ions in Japan in the early 1990s. Although ion beam technology has been used for food crop improvement in China, it has been more extensively used for floriculture plants in Japan. With the aid of IAEA and UNDP, China has been able to develop new, higher-yielding rice cultivars and extend them to farmers across the country as they strive to produce more food from the 33 million ha under cultivation with rice (https://www.iaea.org).

Thus far, over 3000 mutant varieties have been officially released over 60 countries including rice, wheat, barley, sorghum, legumes, cotton, edible oil, ornamental plants, and fruits (www.mvd.iaea.org) [13]. China and India are the major producers of mutant varieties to feed their ever-growing human population. Among all crops, the highest number of mutant varieties released is in rice. In 2005, The International Atomic Energy Agency (IAEA), Vienna, Austria was conferred the Nobel Peace Prize for its contributions to the peaceful applications of nuclear energy in various fields including food and agriculture. The year 2008 marked the eightieth anniversary of mutation induction in crop plants, when an international symposium on induced mutations in plants was organized in Vienna, Austria [14]. The role of mutation breeding in increasing the genetic variability for desired traits in various crop plants have been proved beyond doubt by a number of scientists [15–20].

13.3 GMOs Versus Mutation Breeding Versus Conventional Breeding

When countries reject or ban genetically modified crops over safety concerns, agricultural companies often turn to developing new strains using mutagenesis, wherein plants are subjected to radiation treatments or doused in toxic chemicals that randomly scramble genes to produce new traits.

Is mutagenesis really safer than genetic modification? BioChica, a scientist in molecular genetics addresses the safety differences of GMOs, mutagenesis, and conventional breeding by looking at peer-reviewed academic papers and other publications. A paper from the Proceedings of the National Academy of Sciences, which compared GM rice to "mutant" rice, concluded that, although there are unintended genetic changes in the GM rice, there were far fewer than in rice bred through mutagenesis, although the potential for harm in both cases is trivial. Food safety should be regulated, but the regulations should be on the food product itself, not on the method used to grow a particular crop (www.geneticliteracyproject.org).

GM proponents often compare GM with mutation breeding (or mutagenesis), which they argue that mutation breeding is used by conventional plant breeders and that mutation-bred plants have a history of safe use and do not cause ill health. GM proponents also say that genetic modification is more precise than mutation breeding, and imply that therefore, GM plants should not be regulated any more strictly than those produced by mutation breeding. Once plants carrying radiation-induced mutations have been created, they are crossed with other crop varieties using conventional breeding (the same process is used with GM crop varieties). However, mutation breeding is not in itself conventional breeding (http://earthopensource.org/gmomythsandtruths).

Foreign gene transferring to organisms and producing new traits can cause serious safety problems. For example, in Iran, scientists developed stem borer resistant rice plants via transferring the BT gene to rice but this foreign gene produces toxin in the plant, especially in part "spikes" and this issue endangers human food security. Even, in some cases, transferring a foreign gene can produce allergic interactions, genetic disorders, and different diseases, whereas in mutagenesis, the organism's DNA changes after irradiation and no foreign DNA is entered. In mutation breeding, in order to produce enough and favorable mutant populations, radio sensitivity and postradiation recovery studies must be carried out. LD50 and the optimum dose of irradiation for any explant are determined. Moreover, in this method, many harmful traits are deleted morphologically from the mutant population during the selection process in consecutive years. Also, the nutritional quality of selective samples must be analyzed in different generations of mutation in terms of safety.

Mutation induction techniques can greatly increase the gene mutation frequency and create new germplasm, new materials, and new varieties in a relatively short period of time. The genetic variability increases considerably, by hundreds or a thousand times higher than the natural mutation frequency. The variation spectrum

is wide and various, among them useful variation increases significantly, including some rare mutations that are not easily observed in nature or by the crossing method [13].

13.4 Mutation Breeding Strategy

Plant breeding categorized into three subtypes as mutation breeding, recombination breeding, and transgenic breeding has the potential of generating variation and selection of target lines [9].

Mutation breeding is the process of exposing plant explants to mutagens, physical or chemical agents for inducing genetic variation. It offers good prospects for the domestication of promising underutilized wild species, for agricultural or horticultural uses, as well as for improving adaptation of recently introduced crops to unsuitable environments. One of the most crucial requirements for a successful breeding program is the selection of an effective and efficient dose of a mutagen for mutagenizing the starting material and producing a high frequency of the desirable mutation.

In the case of mutation breeding, the basic fundamental and unique feature is the generation of new mutated alleles. The key steps include analysis of difference in the sensitivity of different genotypes and plant tissues to different mutations often measured using lethal doses (LD), generation of genetic chimeras after mutagenic treatment, and analysis of their effect on transmission of mutated alleles and segregation in the subsequent generation, and also often the recessive nature of induced mutations [9].

The ability to handle large mutagenized populations in a confined space, faster progeny turnover in vegetatively propagated species, and the ability to screen for several biotic and abiotic stress factors in the culture environment make in vitro approaches very efficient. Mutant screening has developed revolutionary changes in the past decade with reverse genetic approaches taking precedence. Therefore, integration of mutation techniques with molecular approaches is providing exciting opportunities for modern plant breeding. Mutation breeding can be enhanced by genetic selection for novel alleles. Through targeted mutation breeding, genotypes with induced or natural mutations in candidate genes are identified for cultivar development. For most horticultural plants, targeted mutation breeding may be a more economically feasible approach to trait development than through transgenic technology.

Like any other scientific innovative technology, mutation breeding has its advantages and limitations. One of the biggest advantages is the creation of new genetic alleles that are not in germplasm pools and the induction of new gene alleles for a commercial variety such that new varieties carrying the desired mutation alleles can be directly used as a commercial variety. Also, the limited genetic changes of any single plant of a mutated population and the often recessive nature enable breeders to develop a new variety in a short breeding cycle. A limitation is

its limited power in generating the dominant alleles that might be desired; its less effectiveness than cross-breeding for a trait needs a combination of multiple alleles, such as tolerance to abiotic stresses. The low mutation frequency requires growing and screening a large population for selection of desired mutants with reasonable confidence [21].

The prime strategy in mutation-based plant breeding has been to upgrade the well-adapted varieties by altering one or two major traits. These include characters such as plant height, maturity, seed shattering, and disease resistance, which contributed to increased yield and quality traits, such as oil profile and content, malting quality, and size and quality of starch granules. For example, short height genotypes in rice, wheat, barley, and maize have contributed significantly to increasing grain yield because of their resistance to lodging and high planting density [22].

A sequential strategy is essential for any mutation breeding steps where mutagenic induction and its mutagenesis are more helpful for autogamous crops than the cross-pollinating one. This is due to several problems regarding the incorporation, selection, and maintenance of recessive mutations in crop plants, many plant breeding problems in the cross-pollinating species, and sometimes many handling-based problems in existing variability. Where the lack of variability exists for specific and simply inherited traits, the basis of choosing between induced mutations and hybridization is essentially the same in self- and cross-fertilizing species. However, the genetic consequences of the failure of recessive imitations to express in crossfertilizing systems without forced selling or sib-mating must be taken into consideration in assessing the cost of such ventures. The efficiency of mutation breeding, more than any other breeding method, is dependent on the effectiveness; useful variants can be recognized in the M2 or M3 generation [9].

In crops where diversity for a given trait is low or nonexistent, induced mutagenesis provides an avenue of possibility.

13.4.1 Mutation Breeding in Seed-Propagated Species

Seeds treated with mutagenic agents give rise to chimeric plants. Chimeric plants produce both mutant and nonmutant seed. This can be problematic; however, one just needs to plant more seeds to find the desired mutants. As long as an efficient screening method is in place, this should produce no significant pitfalls. Mutagenic treatment of seed is by far the most popular method in mutation-breeding programs [2].

13.4.1.1 Mutation Breeding in Self-Fertilizing Species

Breeding mutant traits is fairly straightforward in crops that are capable of self-fertilization. Because many mutations are recessive, after mutagenic treatment, the material should be self-fertilized and advanced to at least the M2 before

phenotypic screening. At this point plants will be segregating for the recessive mutant trait.

Because mutagens act randomly upon the genome, it is important to collect as many positive mutants as possible. This allows the breeder to have a series of lines from which to select for performance in addition to the presence of the mutant trait [2].

After mutagenic treatment, plant materials (seeds, tissues, organs, etc.) and plants grown from them are in the M1 generation; the seeds harvested from M1 plants and the plants grown from these seed are the M2 generation. M1 and M2 populations are populations that are composed of M1 and M2 plants. Their genetic structure is quite different from those of a traditional crossbreeding program, that is, F1 and F2 populations. In diploid plants, the mutation rate per cell is double of the mutation rate per gene. The chance of a simultaneous occurrence of two or more mutations at the same locus of the two homologous chromosomes in diploid plants is rare and so is the chance of segregation of homozygous mutants in the M1 population.

In seed-propagated crops, only germline cells can transmit their genotype into subsequent progenies. Therefore in materials treated with a mutagen, only cells that develop into inflorescences ("initial cells" or "genetic effective cells" in a seed), can transmit mutated alleles into the M2 generation. When a mutation (A→a′) is induced at one of the genes of a homozygous AA locus, the phenotype becomes heterozygous (Aa′). Typically, the mutant allele is completely recessive to the original allele and the Aa′ phenotype cannot be discriminated from the original AA phenotype. Hence, the vast majority of induced mutants cannot be screened in the M1 generation, although there are a few examples of dominant mutations. Mutants that have homozygous mutant alleles (a′a′) will appear in the M2 population as a result of self-pollination of M1 plants. For example, early flowering, semi-dwarf, and male sterile mutants can be visually recognized. There are basically two methods of establishing an M2 population in self-pollinated crops such as barley and wheat. In such monocots the inflorescence is known as a spike [13].

M1-Spike Progeny Method

This is a method in which M2 seeds are harvested separately from each spike of M1 plants. The method was developed by Stadler [8] and has been used effectively by Swedish research groups guided by Gustafsson [23]. Usually 10–20 plants are grown out from seed of individual M1-spikes.

One-Plant-One-Grain Method

The method of constructing the M2 population by planting only one grain (seed) from each plant was proposed as the "one-plant-one-grain method." In practice, the seeds are harvested not from each M1 plant, but from each spike, hence this method

is also known as the "one-spike-one-grain method." If a few seeds per spike are used for the establishment of the M2 population, this approach is called the "one-spike-few-grain method." In mutation breeding, a single target mutant from the entire M2 population is sufficient for utilization and multiplication of the mutants for further selection and testing [13].

13.4.1.2 Mutation Breeding in Cross-Fertilizing Species

Cross-fertilizing species raise some difficulties. Because species that are predominantly cross-fertilizing typically exhibit significant inbreeding depression, the necessary self-fertilizations to identify mutants in the population result in reduced plant vigor due to the genetic background and not necessarily the mutations. This compounds the difficulty of successfully identifying mutations. Crop species with self-infertility mechanisms are especially hard to use mutation breeding methods without elaborate crossing schemes. Because the genetic structure of segregating generations in cross-pollinated crops is quite different from those in self-pollinated ones, the methods for selecting mutants in self-fertilizing species are not applicable for cross-fertilizing species [2].

In some cross-fertilizing (allogamous) crop species (e.g., maize, melon, cucumber, oil palm), male and female flowers are spatially separated. In such species, it is possible to produce M2 populations by artificial selfing of M1 plants by pollinating the female flowers with pollen from male flowers of the same plant. However, homozygous mutants would not appear in such M2 populations, inasmuch as male and female gametes usually derive from different cells in the seed embryo. Because M2 plants are free from chimerism, and therefore homozygous M3 seeds are produced through selfing of heterozygous plants (Aa′) in M2, subsequently homozygous mutant plants are segregated out in M3 populations. In many cross-fertilizing species, however, selfing is not successful due to self-incompatibility systems, and in other cases artificial selfing on a large scale is impractical due to the very small size of flowers [13].

Crossing-Within Spike Progeny Method

The crossing-within spike progeny (CSP) method is composed of: (1) harvesting seeds separately from each spike of the M1 plants or plants in a recurrently treated population; (2) sowing the seeds derived from each spike in a small hill-plot (plot of a small area, e.g., 0.5×0.5 m) in the next generation; (3) isolating each hill from the others by bagging all the plants of each hill-plot just prior to the start of flowering; and (4) harvesting seeds from each hill-plot and sowing them as a hill-plot progeny for the selection of mutants in the next generation. This method is based on half-sib mating, a type of inbreeding that is achieved within each hill-plot from which homozygous mutants will segregate out in the following generation (M3). Under open pollination, the eggs are fertilized with pollen from the other plants. Seed

harvested from the mutated spike are sown in a hill-plot and the plants are bagged just before flowering to avoid fertilization with the pollen from the other hills. Fertilization is performed within each hill-plot. Avoiding chimeric structure within the M1-spike is important in mutant selection in cross-pollinating crops. Because the bagging of each hill-plot just before flowering time in M2 is a laborious task, it is desirable to increase the frequency of the mutated gene in the population in the generation in which bagging is performed. Recurrent mutagenic treatments for successive generations meet these two requirements [13].

Unlike a self-pollinated species, seed sterility does not increase drastically after recurrent treatment in a cross-pollinated species, which was shown in Italian ryegrass after gamma-ray and chemical treatments [24].

In a cross-breeding program, most important traits to be selected are quantitative and, in general, controlled by polygenes. Selections for a specific trait are made in the later generations when most of the loci governing trait are fixed. In a mutation-breeding program, the targeted traits are those usually governed by a single major gene and the selection of mutants is performed primarily in the M2 generation. Seed sterility may be observed and is often caused by the mutagenic treatment. The treatments with radiation often induce chromosome translocations and inversions that lead to pollen and seed sterility. Because either type of induced sterility is more or less inherited by the subsequent generations, hence, if the objective is not to develop mutants for fertility, inflorescences with normal seed fertility should be selected from the M1 plants. In the selection of mutants in self-pollinating crops, the most important point in M3 screening is to evaluate the mutations that have been selected at the M2 generation. The mutation must be highly heritable and fixed in the following generation by selfing the M2-spike progeny in the field. If the undesirable characteristics are associated with a mutant and cannot be removed by ordinary selection, it may be necessary to backcross (BC) the mutant to the original variety and select a promising progeny from the BC population. Generation of doubled haploids from M1, M2, M3, and other mutation generations is a valuable means of producing homozygous mutants and is particularly valuable in species that have a long generation time, for example, perennial trees [13].

13.4.2 Mutation Breeding in Vegetative Propagated Species

Mutation breeding is the only straightforward alternative for improving seedless crops. In other words, mutation breeding is the most suitable method for the breeding of vegetatively propagated crops (VPCs), because the new mutant varieties and the original ones have the same genetic background except for the mutated genes. In VPCs that do not produce any seed (e.g., banana), mutation breeding becomes one of the few available options (other than transformation), inasmuch as cross-breeding is not possible.

All cells exposed to the mutagen will not necessarily incur mutations, but those that do incur mutations will give rise to cells exhibiting the mutation. Identification and propagation of the necessarily large numbers of plants to identify successful mutants is difficult for many vegetatively propagated plants, however, once one is identified, the mutation is fixed in the cloned progeny. Crop species where in vitro techniques exist and can be used to mutate plant material, allow for the regeneration of large numbers of plantlets. This system is highly amenable to both vegetative and seed propagation. A large number of plant species are asexually or vegetatively propagated and are known as vegetatively propagated crops. They include many ornamentals, root and tuber crops, woody perennial and forest trees, fruit crops, and other crops such as peppermint, sugarcane, tea, and many grasses. Cross-breeding of VPCs is often difficult due to various biological limitations, for example, their long vegetative phase, high heterozygosity and polyploidy, incompatibility and other cross barriers, apomixis, and sterility. Mutation techniques can overcome many of these barriers and can be used for the improvement of many VPCs [13].

The possibilities of mutation breeding in vegetatively propagated crops depend on many factors, such as the genetics of the characters involved, the mutagen to be used, the handling of the material after treatment, the availability of a selective screening method, and so on.

The decision as to which breeding strategy (mutation vs. cross-breeding) is appropriate for a specific situation is usually economic: which method is the easiest, the fastest, and the least costly. Effective methods to develop mutants that express phenotypic variation on an individual plant level include: adventitious bud techniques, continuous pruning, grafting and cutting-back techniques, and in vitro culture techniques. Generally, stable mutants are not produced until after several vegetative generations.

In VPCs, meristematic buds are usually used as target material for mutation induction. Many plants possess natural systems of vegetative propagation, especially by tubers, bulbs, rhizomes, stolons, apomictic seed, and so on. In addition, many new in vivo methods, such as stem or leaf cuttings or grafts, and in vitro methods such as cell or tissue culture are also used in commercial production. In practice, the ease of dissociating chimeras after mutagenic treatment plays an important role in the choice of target material. The use of heterozygous starting material (Aa) will be more practical for mutation work than homozygous material (AA or aa), because induced mutations are mostly recessive hence they can be expressed only in plants with the original genotype Aa where the dominant allele is mutated [13].

General considerations for initiating a mutation breeding program of VPCs are:

- The trait for which the variation is sought, and its commercial value/potential, end-user demands
- Genetics of the trait (dominance, recessiveness, pleiotropy, linkage)
- The crop, variety, mode of propagation, degree of heterozygosity, and ploidy level

- Need for mutation induction as an alternative to existing conventional or modern methods
- Plant material to be used for treatment and methods to handle chimerism
- Available information on mutation breeding limitations, if any [25].

Mutation Breeding Steps in VPCs.

In the first year, explants (e.g., shoot meristems or axillary buds) are treated with mutagens with optimal doses. Shoot growth (M1V1 generation) is initiated and assessed for the occurrence of chimera. In the second year, after vegetative propagation of M1V1, M1V2 shoots are assessed for possible occurrence of periclinal or homohistont mutated parts and vegetative propagation of M1V2 shoots is carried out for the isolation of induced mutations. In the third year, genetic uniformity is checked through growth and preliminary evaluation of the mutants (M1V3). In the following years, growth assessment of the mutants is done throughout the vegetative and reproductive stages and evaluation of the mutant's performance for agronomic traits will be fulfilled. The exact number of years needed for assessment varies from plant species to species. Final assessment is carried out in the last MV generation for release as a mutant variety. Inheritance in VPCs is often complex owing to high levels of heterozygosity and ploidy, which make genetic analysis difficult [13].

13.4.3 Ploidy and Mutation Breeding

Mutagenesis of polyploid plant species is difficult. Because most mutations are recessive, plants must be homozygous to display the trait. Polyploidy conditions can further complicate the process of reaching homozygosity for the mutation, so selfing must be carried out in additional generations to ensure presence of the mutation [2]. They can more efficiently repair damage to their DNA. The ploidy level of the target species also influences mutation response. Ploidy deficiencies include a reduction in genome number, for example, from diploid barley to haploid barley. Haploid production and thereby doubled haploid production by inducing embryogenesis in haploid (gametic) cell cultures to produce homozygous lines is a valuable technology in plant breeding and genetics of many species.

Polyploidy either by genome duplication (autoploids) or genome addition (alloploids) has occurred naturally in the evolution of many species and has also been induced for crop improvement. One effect of polyploidy is to increase the volume of the nucleus; this in turn increases cell size and tissue, organ, and plant size [13].

13.5 Molecular Mutation Breeding

The term "molecular mutation breeding" is defined as mutation breeding in which molecular biological knowledge, techniques, and tools are used. Experimental mutagenesis of the early twentieth century led to unprecedented breakthroughs in plant breeding; this, however, was based largely on phenotypic selection and without much knowledge of the genetic controls of target traits. Techniques in molecular genetics are rapidly evolving and methods in handling the large datasets produced (bioinformatics) are expected to have a massive impact on molecular mutation breeding. High-throughput DNA technologies for mutation screening such as targeting induced local lesions in genomes (TILLING), high-resolution melt analysis (HRM), EcoTILLING, and so on are the key techniques and resources in molecular mutation breeding. Molecular mutation breeding will significantly increase both the efficiency and efficacy of mutation techniques in crop breeding [9].

Induced mutagenesis was almost irrelevant to reverse genetics before the development of TILLING and similar generic reverse genetics strategies. This approach can be used both for functional genomics and practical breeding. In plant genomics research, in addition to identifying the function of a particular gene in a given plant species, the effect of various mutant alleles can be assessed using TILLING technologies. Starting with a homozygous population is desirable. From a technical point of view, the TILLING protocol includes four main phases: (1) generation of a mutant population, (2) selection of target genes (DNA preparation and pooling), (3) molecular screening, and (4) recovery of mutants [13].

13.5.1 De-TILLING

Fast neutron mutagenesis often results in kilobase-scale DNA deletions. As a new knockout technique to obtain deletion mutants for target genes, a strategy to screen for rare deletion mutants in large fast neutron mutagenized populations was first developed by Li et al. [26, 27] and demonstrated in *Arabidopsis* and rice. It combines fast neutron mutagenesis and high-throughput PCR screening, named "Deleteagene" (delete-a-gene). This strategy has been further developed and named deletion-TILLING, or de-TILLING [28].

The de-TILLING method includes three key technological aspects:

1. Fast neutron mutagenesis, which generates DNA deletions in different sizes
2. A DNA pooling strategy to reduce the number of PCRs needed
3. Technologies that allow a mutant allele, possessing an internal deletion, to be amplified in pools with excessive genomic target sequence [13].

13.5.2 New Platforms for TILLING

Typical or simplified TILLING systems are based on the hetero-duplex cleavage by endonucleases such as Cel1. There are instruments that can be used to differentiate hetero-duplex from homo-duplex, hence no cleavage is needed. Using CSCE or HRM, the only step required is a simple PCR before either capillary electrophoresis or DNA melting curve analysis [13].

13.5.2.1 CSCE-Based TILLING

CSCE is a nonenzymatic differential DNA conformation technique for SNP discovery. After PCR amplification, and the denaturing and reannealing of amplicons, several duplex species are formed, for example, homo-duplex of wild type (WT-WT) and mutant (M-M) and hetero-duplex of WT-M. Because of the mismatch formed in the hetero-duplex, it migrates at a different speed from the homo-duplex during electrophoresis in capillaries filled with CAP, a semi-denaturing polymer, thus allowing the identification of pools containing a mutation within the target fragment [13].

13.5.2.2 HRM-Based TILLING

Similar to CSCE, HRM is also a nonenzymatic mutation screening technique; it reveals sequence variants due to distinct patterns in DNA melting curve shape. It has been used recently in many ways as a novel approach to study genetic variation in many fields with applications ranging from qualitative SNP detection to semi-quantitative analysis of methylation. When using equipment and reagents such as the Light Cycler® 480 System and its accompanying High Resolution Melting Master mix, which contains a saturating fluorescent dye, realtime PCR is carried out using a touchdown protocol, with annealing temperatures ranging from 70 to 60 °C [13].

13.5.3 EcoTILLING

The genomes of individuals within a single species contain significant genetic variation that has arisen from spontaneous mutation. The vast majority of this diversity is in the form of single nucleotide changes commonly referred to as simple nucleotide polymorphisms (SNPs). Such naturally occurring SNPs are of great interest to scientists because they are useful as genetic markers in mapping, breeding, and genotyping and can provide information concerning gene structure, linkage disequilibrium, population structure, or adaptation [9]. Both nucleotide

changes and small insertions and deletions are identified, including at least some repeat number polymorphisms. This method is called EcoTILLING. The technology is applicable to any organism even including those that are heterozygous and polyploid [29].

13.6 Application of Mutation Breeding in Improvement of Quantitative Traits of Plants

Mutagenesis for resistance to abiotic stresses is a well-known effective and efficient breeding approach in order to create new desirable genetic variability, as the use of the traditional breeding methods have narrowed genetic variability in the cultivated crop species over a long period. A quantitative trait mutant cannot be detected with a high level of confidence owing to its interaction with environmental factors, therefore a different procedure is recommended for selecting such mutants. If the heritability of the targeted quantitative trait is low and is much influenced by environmental effects, mutants should be screened from the M3 population based on the mean phenotypic value of progeny derived from M2 spikes or plants. For a quantitative trait, normally M3 families and subsequent generations are used for phenotypic screening. When screening for a quantitative trait, such as yield, disease resistance, abiotic stress tolerance, or quality, mutant selection should be postponed until the M3 generation [13].

In India, breeding work for salinity resistance is mainly carried out at the Central Soil Salinity Research Institute, Karnal (http://www.agriinfo.in). Also, in Bangladesh, breeding for crop quality and abiotic and biotic stress tolerance is mainly carried out by the Bangladesh Institute of Nuclear Agriculture with the collaboration of IRRI (http://www.bina.gov.bd). Thus far, two salt-tolerant rice varieties (BINA dhan 8 and BINA dhan 10) and two submergence-tolerant rice varieties (BINA dhan 11 and BINA dhan 12) have been developed by BINA.

In the United States, there are firms such as Arcadia Biosciences that specialize in TILLING, and a number of crops are currently being developed around the world, including salt-resistant tomatoes, drought-resistant soya beans, and strawberries with a longer shelf life, gluten-free cereals, fungus-resistant barley, and yellow tomatoes (http://www.gmo-safety.eu/News).

13.6.1 Mutagenesis for Tolerance to Drought Stress in Plants

In recent years, drought has occurred more and more commonly as a result of global warming and climate change. The plant traits improved by mutation breeding include: yield, flowering and ripening time, adaptability, plant type and growth

habit, resistance to lodging and stem breakage, shattering and shedding resistance, tolerance to temperature, drought, heat, and salinity [30].

13.6.1.1 Probability of Obtaining Mutants

Often a few loci possess significantly higher genetic effects. Mutations at a gene at such loci with a large genetic effect can be selected after mutagenic treatment. The occurrence of a grain carrying a mutant gene with the low probability of only 10^{-12} per gene per generation due to a spontaneous mutation will still amount to several thousands of independent occurrences (and thus grains carrying the mutant gene) worldwide [31].

It is important to determine the most efficient sizes of M1 and M2 populations to ensure a reasonable probability of identifying a desired mutant. Factors such as chimerical nature, harvesting and growing practices, and the genetic nature of traits of interest can all significantly affect the population size and hence the efficiency in mutant development. A quantitative trait such as yield or quality of grains is generally controlled by many genes and, in addition, influenced by environmental factors. The mutation rate of a gene is very low, therefore the chance of simultaneous occurrence of mutations at two or more genes is negligible. Hence the selection of mutants in mutation breeding is usually unsuccessful for a quantitative trait, particularly when the number of loci controlling the trait is many and the effect of each locus is small as compared with environmental variation. But many quantitative trait locus (QTL) studies revealed that the genetic effect among the contributing loci is not equal [13].

As mentioned above, determination of the target population size in M1 is the most crucial component of mutation breeding. It is obvious that the population size will depend on the inheritance pattern of the gene. If the mutation is monogenic recessive, the probability of recovering a mutant phenotype will be higher than for a trait controlled by more than one gene. In practice, 10 times of the size has to be considered, because the mutation produced may be useful or undesirable. Mutation breeding is an input-intensive process. It is therefore advisable to select mutagens with high mutation frequency, so that Ml generation size can be reduced. It is to be remembered that germline mutations take place only in the initial cells of the embryo, therefore depending on the nature of the species, products of initial divisions should be screened. For example, cereals such as rice, wheat, barley, oat, and the like produce multiple tillers. Those tillers that generate first (primary tillers) have the maximum chance to carry a mutation. In the case of tuber crops such as potato, the mutation may be present in any of the stems arising from different discs of a tuber, therefore each of them has an equal chance to give rise to a mutation. Genetically, a mutant plant in Ml should be heterozygous, because during treatment only one allele is affected by one mutation. Only dominant mutations can be identified [9].

13.6.1.2 Determining an Optimal Treatment Dose

Mutation frequency usually increases linearly with an increasing dose of mutagenic treatment, but survival and regeneration capacity decrease with increasing dose. The radiosensitive curve should be determined to calculate LD50 dose (lethal dose) for each experimental plant to avoid either very high or very low dosage. Moreover, plants and even varieties differ in radiosensitivity [32]. In order to obtain the highest of favorable mutations, it is necessary to determine LD50 and optimum dose. This dose is a dose that causes the maximum favorable mutations with minimum damage to the plant.

In seed-propagative plants, seed is the main material for mutagenesis in most cases. In vegetative-propagative crops, when determining an optimal dose for treatment, these two aspects should be considered. In VPCs no meiosis is experienced throughout the process of mutant development, therefore, the genetic constitution of a mutated cell is inherited by its lineage cell lines and plants unchanged. Therefore, it is impossible to separate useful mutations from unwanted ones that occurred in the same cells. This is in sharp contrast to seed-propagated crops, where unwanted mutant genes can be separated from desired ones through self-crossing or backcrossing. Therefore, a dose lower than LD50 is preferred in mutation breeding of VPCs.

13.6.1.3 Management of Early Generations of Mutation

The treated seeds need to be handled with care. The seeds treated with physical mutagens can be stored before sowing. However, the seeds treated with chemical mutagens should be washed thoroughly and be planted as soon as possible. The time of sowing should be slightly later (2 or 3 weeks) than normal so as to reduce excessive vegetative growth. The purpose of isolation of the M1 is to avoid the introduction of genetic variability other than that induced with the mutagenic treatment. Mechanically isolation can be achieved by bagging spikes in cereals using plastic or paper bags to prevent cross-pollination and bird damage. Even if the objective of a mutation project is to select mutants with enhanced tolerance to drought or salinity, the M1 plants should be grown in a nonstressed condition; otherwise, there will be insufficient number of plants generated in the M2. M1 plants should be grown either at a reasonable distance from other varieties (physical isolation) or in a time period when no other plants would flower simultaneously (biological isolation). In the case where the seed yield from each branch is reasonably adequate, it is suggested that each primary branch may be harvested separately. In the case of cereals, the individual plant or spike can be harvested [33, 34].

Many mutants with desired traits are selected in the second or third generation after mutagenic treatment and subsequently released as new cultivars after agronomic evaluation in regional and national trials.

In seed-propagated crops, sowing of the M2 generation depends upon the method of harvesting of the M1 generation. Two methods of sowing the M2 generation can be followed. First, M1 plant to row, where all seeds produced from a single plant are grown in a row. The success of its use will depend, to a large extent, on how well the branching has been controlled because it tends to dilute the yield of M2 mutants. The second method is of M1 spike or branch to row, which offers the greatest precision with regard to the origin of a mutant when the material treated is genetically homogeneous as regards the nonmutant allele and when outcrossing is controlled.

From M1 to M3 through from germination to harvest, the mutated plants are carefully observed for all viable mutations. The plants with different morphological traits are isolated for chimeric and dominant mutations through their life period in M1, and they are isolated for recessive mutations in M2 and M3. All kinds of morphological mutants isolated in M1 and M2 are confirmed for true mutations in M2 and M3, respectively. Individual plant selections based upon phenotypic variations are started in the segregating M2 population, focusing on agronomic and yield characters [9].

In vegetative-propagated crops, selection for interesting mutants can take place three to four years after irradiation. In this way, some commercial mutants have been obtained [35].

Visual selection of the mutant phenotypic variation is the most effective method of selection in VPCs. It can be used effectively for identifying common traits and characteristics such as color changes, plant morphology, earliness, resistance to pests and diseases, and so on. Selection usually starts from M1V2 and continues for confirmation in M1V3 or M1V4 generation. Because the probability of identifying desired mutants in a homogeneous M1V2 population is quite low, selection is necessary in the M1V3 generation or in some cases in the M1V4 generation [13].

Mainly three types of screening/selection techniques can be employed for the selection of mutants in M2 and subsequent generation via visual, mechanical/physical, and other methods [36].

Visual screening is the most effective and efficient method for identifying mutant phenotypes. Visual selection often is the prime basis for selecting for disease resistance, earliness, plant height, color changes, ion-shattering, adaptation to soil, climate, growing period, and so on. Mechanical or physical selection can be used very efficiently for seed size, shape, weight, density, and the like, using appropriate sieving machinery. In other categories, chemical, biochemical, physiological, or physiochemical-like screening procedures may be needed for selecting certain types of mutants [9].

13.6.1.4 Screening and Selection of Mutants for Resistance to Drought

A quantitative trait mutant cannot be detected with a high level of confidence owing to its interaction with environmental factors, therefore, a different procedure is recommended for selecting such mutants. Selection of mutants on the basis of the

mean value of M2-plant–derived progeny in the M3 instead of the value of the M2 plant is a possible solution. If the heritability of the targeted quantitative trait is low and is much influenced by environmental effects, mutants should be screened from the M3 population based on the mean phenotypic value of progeny derived from M2 spikes or plants [13]. The mutants confirmed in M3 are screened for resistance to drought and heat in field and greenhouse experiments. In the drought-resistant experiments, the mutants are selected for the background of their parents. In other words, the mutants are used according to the reaction of parents to abiotic stresses, and compared with their parents and international standard cultivars (ICARDA).

In these experiments, chlorophyll content (CC), drought resistance score (DR) using a 1–9 visual scale, root (RL) and shoot lengths (SL), plant height (PH), canopy width (CW), biological yield (BY) per plant and seed yield (SY) per plant, 100-seed weight (SW), and harvest index (HI) are studied. CC is recorded using a chlorophyll meter. Of course, the evaluation scale of drought resistance (tolerance) in vegetative stages is different and depends on the kind of plant species and sensitive stage of plant growth. For example, in rice, the drought tolerance scale is on leaf rolling score (0–9) at the end of the vegetative stage. Yield is another screening scale that is estimated in all plants after finishing of drought stress, usually at the end of the reproductive stage. In rice, the most important factor of yield is spikelet fertility. Therefore the drought tolerance level of mutant rice plants is estimated on a 1–9 scale of spikelet fertility.

13.6.1.5 Evaluation of Preliminary Yield of Promising Drought-Tolerant Mutant Lines

Yield traits are the most important criteria for water-deficit tolerance screening [37–40]. In rain-fed paddy fields, water shortage has been well known as being a seri-ous issue, especially in the reproductive stage, during which plants are particularly sensitive, leading to low crop yield [37, 39]. The flowering stage is the most drought-sensitive stage as drought stress during this stage results in loss of yield due to low spikelet fertility and low full seeds. In vegetative-propagated species such as sweet potato, bulk irradiation is done at respective optimum dosages and explants are propagated in vitro up to M1V4 and M1V5 stages to dissolve chimeras and obtain stable mutations.

Promising mutant lines are further identified and evaluated in a replicated preliminary yield evaluation trial. Also, in seed-propagated species such rice, selective mutant lines are grown up to M4 and M5 to achieve genetic stability and enrich germplasm by selecting genotypes with superior qualitative and quantitative traits. Preliminary yield of promising drought-tolerant mutant lines is evaluated in completely random design in three replications [41].

13.6.1.6 Assessment of Adaptability and Stability of Promising Drought-Tolerant Mutant Lines in Advancing Generations

Usually, this evaluation is carried out in different ecological regions for at least two years. The assessment method of promising mutant lines is approximately similar to other native genotypes and cultivars.

A randomized complete block design with three replications is applied. In seed-propagated species such as cereal, promising mutant lines with control and commercial cultivars are sown on plots randomly. With attention to lack of purity and homogeneity of genotypes and in result, different propagative and reproductive specifications especially in open-pollinated species, spaces between mutant plants in plots are variable on studied mutant genotypes and cultivars. All agronomic practices recommended for production are applied equally for each plot. Plot base and individual plant base data are collected. Common traits that are evaluated include percentage of fertility, plant height, flowering date, and yield, especially in plot base. All the data are collected when the crop reaches physiological maturity. These data are subjected to analysis of variance (ANOVA) using statistical software. The data are combined over locations after carrying out analysis of variance for each location separately, and homogeneity tested as suggested by Gomez and Gomez [42]. Means are separated using Duncan's multiple range (Duncan) test. Environment interaction of the genotypes in biplot analysis is conducted using GenStat 15.1 computer software.

13.7 IAEA/FAO Activities on Developing Drought-Tolerant Plants

The International Rice Functional Genomics Consortium announced the public availability of more than 200,000 rice mutant lines, which represent mutations in about half of the known functional genes mapped for rice to date [43].

The database maintained by the UN Food and Agriculture Organization and the International Atomic Energy Agency contains only around 3000 such plant varieties, and this number includes not only food crop plants but also ornamental plants. It also includes not only the primary mutant varieties generated through mutagenesis, but also any varieties that have been created by crossing the primary mutant varieties with other varieties by conventional breeding. Thus the actual number of primary mutant varieties is a fraction of the 3000 varieties listed in the database. These varieties have been officially released in over 60 countries including rice, wheat, barley, sorghum, legumes, cotton, edible oil, ornamental plants, and fruits (www.mvd.iaea.org). Drought-resistant varieties have been developed in several crops in many countries throughout the world. In India, drought- and salinity-resistant or tolerant varieties have been developed in several crops such as cotton, rice, and sugarcane. In cotton, drought-resistant varieties have been

developed through induced mutations. In wheat, drought-resistant varieties have been developed in China and the USSR through induced mutations. In *Luthyrus saliva*, a drought-resistant variety has been developed by induced mutations in USSR (http://www.agriinfo.in). The group in Vienna, according to program head Pierre Lagoda promotes developing more "sustainable" crops by irradiating them to resist threats such as drought, insects, disease, and salinity [11].

The Mutation Breeding Project focused on the improvement of drought tolerance in soybean and sorghum represents one project developed by the Forum for Nuclear Cooperation in Asia (FNCA) in 2002. Ten drought-tolerant mutant lines of sorghum were obtained in Indonesia. In dry seasons, these lines have biomass production and grain yields significantly higher than original variety Durra and the national check variety [44]. Eight drought-tolerant pure soybean lines were selected in Malaysia. Four mutant lines (GH-7, I-209, M-220, and 60-MBB) were already distributed to field test in Malaysia and the Philippines. In the Philippines, five promising drought-tolerant soybean lines were developed. In Vietnam, the results of a local adaptability test at five stations showed that the line D.96 adopted as a national variety in 2004 and named DT96 was tolerant to drought and high yielding [44].

In Iran, Hallajian et al. [45] produced several new promising drought- and salt-tolerant lines in rice. Also, these promising lines had superior qualitative and quantitative traits such as early flowering, dwarfing, high aroma, resistance to herbicide and lodging, high yield, and so on. Now these promising lines are being evaluated in different geographical sites of Iran in order to introduce, register, and release new elite rice cultivars (www.aeoi.org.ir; Fig. 13.1).

In Australia, there have been some reports in improving some rice varieties for their tolerance to some adverse environmental conditions and higher grain yields (IAEA 1980). Characterization of ethyl methane sulfonate (EMS) induced mutants of N22 for water stress and heat tolerance was reported by Panigrahy et al. [46]. Nagina 22 (N22) is a deep-rooted, drought- and heat-tolerant aus rice cultivar.

Scientists from the University of Agricultural Science and BARC produced a large seed variety of groundnut. Hundreds of farmers are producing even up to 7 tons/ha of some varieties of groundnuts in some states. A drought-tolerant early maturing variety and an early maturing large seed variety of groundnuts are being cultivated in large desert areas in Rajasthan [12].

In West Africa, sorghum is also undergoing irradiation treatment and, in field trials, some of the new mutant varieties produced have demonstrated increases in yield of 30–50 %, higher protein content, and earlier maturation compared to local cultivars. Some varieties demonstrated an improved tolerance to drought and the new plants also maintained the important characteristics favored by farmers (www.new-ag.info).

Fig. 13.1 Drought stress in the field and promising drought-tolerant line (2013)

13.8 Conclusion and Future Perspectives

With the imminent threats posed by global climate change to crop production and the ever-increasing and more sophisticated demands of agricultural products, crop improvement efforts have to be more powerful and precise in developing new crop varieties. Breeders therefore require tools that permit achieving subtle changes to the genetic make-up of otherwise superior crop varieties, for example, high yielding but lacking in specific quality traits, and yet leaving the genome largely intact in order not to disturb already stacked alleles of genes. The spectrum of available mutation techniques has significantly increased; as a result, following the recent trend in the release of crop mutant varieties in some countries, the number of officially released mutant varieties listed in the FAO/IAEA Mutant Varieties Database will expand exponentially year after year. The conventional breeding method takes several years to develop a new cultivar/variety from wild species. Induced mutagenesis and its breeding approaches are potential tools and are being highly used in crops to improve their quality and quantitative yield traits.

The availability of genomics information in the public domain coupled with recent advances in molecular and cellular biology techniques have paved the way for transforming old mutation techniques into state-of-the-art technology for both crop improvement and basic genomics research. Some technologies are already in place and when integrated into mutation research, they will greatly increase the efficiency and application of mutation techniques in plant research. For example, the next-generation sequencing technologies, such as the Roche 454 Genome Sequencer-FLXFe Sequencer-FLX Biosystems SOLiDF™ instruments, have the potential to reduce the cost of genome sequencing by several magnitudes, and simplify the process of mutation detection, the key point in mutation research and application programs. In particular, they will enable the identification of mutant genes underlying important quantitative traits such as drought tolerance and yield, something that is still very difficult if not impossible with traditional means (www.iaea.org).

Most of the mutant lines were found to be more resistant to the concerned stresses than their parents and the best international checks. These mutants will be used either (1) directly in the target environments as commercial varieties, or (2) indirectly in breeding programs as useful parents. Thus, mutation-assisted plant breeding will play a crucial role in the generation of “designer crop varieties” to address the uncertainties of global climate variability and change, and the challenges of global food insecurity [9]. Our present knowledge about mutation breeding and drought tolerance in plants indicated that mutation breeding can be applied as a useful tool in developing new drought-tolerant genotypes and cultivars with superior traits and can help to develop cultivation of different plant species in drought periods or low falling regions of the world.

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