Mutations: In Crop Evolution, Improvement and Reverse Genetics

AK Sharma[1], Sangita Bansal, RS Sengar and Vipin Kumar*
Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh (India)-250 110
[1] *Present Address: Ramie Research Station (ICAR), Sorbhog, Assam-781 317*

Mutation has been used as efficient tool for plant improvement and is also considered as one of the important factors involved in crop evolution. Spontaneous mutations known to occur in nature have led to development of new cultivars. During the last few decades it has become an effective tool in reverse genetics. The paper reviews the various roles of mutation in crop evolution, plant improvement and reverse genetics.

Key Words: Mutations, Macro evolution, Reverse genetics

Most efficient tool available to plant breeders in their quest to develop improved cultivars and a scientific cause of crop evolution is mutation. A mutation is a change in the structure as well as function of a gene. In most cases, it is deleterious as it means that the gene doesn't produce what it should. However, mutations can result in valuable new traits. Spontaneous mutations occur in nature at a relatively continuous and frequent rate. In almost all the crops species, large numbers of spontaneous mutations have been recorded and used either directly as new cultivars or breeding line in breeding programs. In all vegetatively propagated crops, mutations are being used successfully for developing new cultivars (*e.g*. changes in fruit color or time of fruit maturity, etc). Recent decades have witnessed intensive work on the induction of mutations by using irradiation, chemicals and other mutagenic agents. The frequency of induced mutations almost doubles those occurring naturally and they have been looked on as a powerful tool for the development of new cultivars. However, available mutagens cause not only changes in genes but also chromosomal aberrations, many of which are deleterious in their effect on the trait and on the entire organism. Consequently, there have been a limited number of induced mutations directly usable as new cultivars. Since mutation particularly spontaneous mutation is an ultimate source of variation and speciation in entire living organisms that ultimately resulted in natural evolution of cultivated species from their wild sources. In the mid of 19th Century induced mutation became a most effective tool for crop improvement and in the beginning of 21st century mutations has became an efficient tool in the reverse genetics.

Mutations in Crop Evolution

Biological evolution is the natural process that explains the common relationship of all life on earth now and in the past. Evolution is a result of the combined changes that occur in a **population** of **organisms** over successive generations. All living organisms can **inherit** these changes because they have genes, which are made up of molecules called **DNA**. Changes in these molecules, called (**mutations**), can become a new feature in the offspring of a living organism. These new features called **traits** are almost always minor. Because none of the organisms have exactly the same traits, they will live and reproduce differently, some more effectively than others. Scientists call this process **natural selection**. Over the time, the favorable trait will become common in the descendents of the creatures. Evolution is a continuous process, resulting from accumulation of minor mutations most of which exert only small effects.

Mutation
Gene Flow
Genetic Drift **+ 3.8 billion years = Macroevolution**
Natural Selection

Though inconsistent in his views, Darwin also considered that the slight and fluctuating variations are of more importance. Baur (1924) repeatedly emphasized greater importance of small mutations in evolution. Most of the evolutionists like Stebbins (1950) and Dobzhansky (1951) recognized the small steps in form of minor mutations as the most significant steps of evolution and speciation. Crop plant domestication began approximately 10,000 years ago at the dawn of agriculture (Harlan, 1992). During the domestication process, early agriculturists consciously or unconsciously selected among wild germplasm for material that was better adapted to human use and cultivation. Since the transition from wild species to domesticate, crop plants have continued to change due to selection exerted by ancient and modern plant breeding and cultivation practices. These changes

* *Author for correspondence: E-mail: vipinch1@yahoo.co.in*

that occurred subsequent to the initial domestication event(s) are known as crop evolution. As a result of both crop domestication and evolution, today's domesticated species are differentiated from their wild ancestors by an assortment of morphological and physiological characteristics. This is true despite the often large differences in gross morphology between crop and their progenitors because artificial (human) selection is likely to have acted primarily on the small sites of genes controlling morphogenetic traits of interest to humankind, leaving the vast majority of genome to evolve at much slower pace.

Mutations in Crop Improvement

The selection of better natural genetic variation and creation of new such variations followed by selection of desirable ones are the major activities in plant breeding. New genetic variability can be created through conventional and non-conventional breeding methods, namely, introduction, selection, hybridization, polyploidy, mutation breeding, etc. According to Brock (1971) induced genetic variation represents a more efficient source of genetic variability in the gene pools conserved by nature. Since genetic variability is a prerequisite for any successful breeding programme, the creation and management of genetic variability becomes central base for improvement of any crop species. Selection and hybridization can be used as conventional methods for the improvement of qualitative as well as quantitative traits, but in a highly self-pollinated crop, these methods with a limited genetic variability do not prove so effective. Spontaneous mutations occur in natural population and give rise to important natural variations. Part of the possible variations arising in a plant population is due to spontaneous mutations, which mostly arise at a very low frequency of $10^{-5} – 10^{-8}$ per locus, and for various reasons often remains undetected and unexploited. Therefore, spontaneous mutation can not be expected to serve the cause of crop improvement effectively. In this context, it is quite desirable to select and apply induced mutagenesis, which is recognized as a quick and successful method in creating genetic variability and bringing about desirable improvement.

A very good example of spontaneous and induced mutations is the one which resulted in green revolution in Asia and Africa. There are many genes associated with a semidwarf growth habit in wheat (Ellis *et al*., 2005). They are known as *reduced height* (*Rht*) genes, and many of them are dominant or semidominant, indicating that they actively inhibit growth through a so-called gain-of-function mutation. Peng *et al*. (1999) found that the *Rht-B1b* and *Rht-D1b* dwarfing alleles each contained a point mutation that introduced a stop codon into a conserved region known as the DELLA domain, which is present near the N-terminus of the protein. Peng *et al*. (1999) proposed that, in *Rht-B1b* and *Rht-D1b*, translation of the protein might restart after the introduced stop codon, resulting shorter proteins in which part of the DELLA domain has been lost. These proteins would then be resistant to GA-induced degradation. As yet, this proposal has not been confirmed experimentally, and there are many uncertainties still to be resolved about the *Rht* genes. For example, two further dwarfing genes, *Rht-B1c* and *Rht-D1c* (formerly known as *Rht3* and *Rht10*, respectively), produce a more severe dwarf phenotype than *Rht-B1b* and *Rht-D1b*. As their name indicates, *Rht-B1c* and *Rht-D1c* are allelic to *Rht-B1b* and *Rht-D1b*, respectively, but it is not known which mutations they contain and how these might result in more severe dwarfing. The rice dwarfing gene arose as a spontaneous mutation in the Taiwanese *indica* strain woo-gen. The resulting strain dee-geo-woo-gen was used in breeding programs in eastern Asia to produce many of the high-yielding semidwarf cultivars grown today. The *sd1* allele in dee-geo-woo-gen contains a 383-base-pair deletion, which introduces a stop codon so that a truncated, inactive enzyme would be produced. Remarkably, semidwarf lines produced independently by mutagenesis, such as the *japonica* varieties Calrose 76 and Reimei, were found also to contain mutations at the *sd-1* locus. Thus, the ideal combination of short stature and high yields provided by *sd-1* mutants has ensured that these alleles have been selected consistently despite the availability of numerous other dwarfing genes in rice, many of which affect GA biosynthesis.

The induced mutations have a great potential to improve any crop species or to create desirable genetic variability, therefore induced mutation is considered as easy, rapid and effective tool of plant breeding. Artificial mutation may be induced following treatment with certain physical or chemical agents called mutagens. In most of the cases, only six mutagens, namely, X- rays, gamma rays, neutron in physical mutagens and EMS, sodium azide and MMS in chemical mutagens are commonly used to create genetic variability in plant improvement. The idea of producing mutation artificially and using it for breeding purposes was clearly started as early as 1901 by De Vries. Induced mutagenesis has been used as a

supplement to conventional breeding procedures ever since evidences on induction of heritable changes in animals (*Drosophila*) and plants (maize and barley) with X-rays were presented by Muller (1927) and Stadler (1928), respectively. They provided the first scientific proof that ionizing radiations like X-rays can enhance the frequency of mutations in animals as well as in plants. Subsequently, studies by Auerbach and Robson (1947) introduced chemicals in the field of mutation breeding. By mutations, plants can be genetically modified and improved in a way similar to traditional breeding methods. The first and the most extensive study involving deliberate selection of mutations in a quantitatively inherited character following X-ray irradiation comes from Gregory's work (1955, 1956(a&b), 1965 and 1968) on peanuts. He presented data to support the hypothesis that as the magnitude of phenotypic effect of the mutation decreases, the frequency of mutant plants increases. Gradually, it has been recognized that micromutation might play an important role in plant breeding, and studies on this aspect for different quantitative characters in mutagen treated populations assumed importance. The parent material for mutation breeding programme can be a recently released cultivar or a promising line. Stubbe (1937) described the small mutations in higher plants for the first time, whereas Knapp and Schreiber (1939) gave clear suggestions for the utilization of micromutations in plant breeding.

The period after 1950 has witnessed many useful mutations for both qualitative and quantitative traits in important crops, especially food crops. During this period, not only the number of released mutant crop varieties increased greatly but the proportion of varieties developed through cross breeding using induced mutants also increased tremendously indicating the recognition of induced mutants used as parents in the breeding programmes by the breeders (Leenakumari et al., 2000). The success achieved with mutation breeding techniques in major crops like rice, wheat and barley during the last half a century has proven that it is no longer a controversial breeding methodology, but should be considered as an important technique to complement conventional breeding technology (Sharma, 1986). Pure and healthy seeds are generally used as the breeding material. Seeds have the advantages over the other plant propagates; because they can be mutagenised in different physical environments, can be desiccated, soaked, heated or frozen or can tolerate high pressure and vacuum conditions. There are more than 2,252 cultivars obtained either as direct mutants or derived from the crosses have been released worldwide in 50 countries (Maluszynski and Ahloowalia 2000) in a number of crop species. Out of the total varieties released in the world 60% were released from 1985 onwards. Most of the mutant varieties were released particularly in China (26.8%); India (11.5%); USSR and Russia (9.30%) (Ahloowalia et al., 2004). Many induced mutants were released directly as new variety, other were released as parent to drive new variety. The maximum number of varieties has been developed in rice followed by barley and wheat through mutation breeding. Discovery of spontaneous mutants or sports by observant growers has been an important means of cultivar improvement in many fruit crops such as apples and citrus. For example, the apples 'Royal Gala' and 'Imperial Gala' are natural mutations of `Gala'. In kiwifruit, it is more difficult to trace the source of fruit variants because of the replacement cane pruning system. Unusual fruit found in the packing shed one season may not reappear on the vine the following season if the mutation occurred on a single cane removed during normal winter pruning. Desired improvements/ differences would be difficult to detect on single canes. Nevertheless, natural mutations of `Hayward' kiwifruit have been observed in orchards in New Zealand. Gamma irradiation of dormant budwood has been used in New Zealand in an attempt to induce useful mutations in 'Hayward'.

Mutation in Reverse Genetics

The 21st century is considered as an era of biotechnology and information technology. In the era of biotechnology the genetic engineering has been progressed and strengthened through the discovery of array of molecular techniques *viz*., PCR, antisense RNA technology, terminator and verminator, genomics and proteomics etc. With the onset of these tools and techniques, a new and innovative technique *viz.* TILLING (Targeted Induced Local Lesions in the Genome) was developed which will be a potential technology in the reverse genetics. Reverse genetics aims to identify the function of a gene with known sequence by phenotypic analysis of cells or organisms in which the function of this gene is impaired. Commonly used strategies for reverse genetics encompass transposon mutagenesis (Tissier *et al*., 1999) and RNA-mediated gene silencing or RNA interference (Voinnet, 2002). Most of the genes of an organism are known from sequence, but most of the phenotypes are obscure. Thus, reverse genetics has become an important goal for many biologists. However, reverse-genetic methodologies are

not similarly applicable to all organisms. In the general strategy for reverse genetics that is called TILLING, traditional chemical mutagenesis followed by high-throughput screening for point mutations. TILLING promises to be generally applicable. Furthermore, because TILLING does not involve transgenic modifications, it is attractive not only for functional genomics but also for agricultural applications.

Reverse genetics has become an important goal for many biologists, and new technologies are in great demand (Nagy *et al*., 2003). Large-scale DNA sequencing projects have changed the way that biology is performed. The traditional pursuit of a gene starting with a phenotype has given way to the opposite situation: most of the genes are known from sequence, but most of the phenotypes are obscure. However, unlike genomic technologies such as DNA sequencing and BLAST searching, reverse-genetic methodologies are not similarly applicable to all organisms. For example, T-DNA insertional mutagenesis has turned the problem of obtaining a gene knockout into an in silico procedure for >70% of Arabidopsis genes (Alonso *et al*., 2003), but no comparable resources exist for rice (*Oryza sativa*) or maize (*Zea mays*), despite the increasing availability of high-coverage genomic sequence. RNAi-based silencing is an exciting strategy for reverse genetics (Waterhouse *et al*., 1998); however, throughput is limited by the difficulty of delivering siRNAs to target loci. Furthermore, the promise of using these reverse-genetic technologies for crop improvement is hampered by genetically modified organism issues.

In TILLING, traditional chemical mutagenesis is followed by high-throughput screening for point mutations. Because of the wide use of chemical mutagenesis for forward-genetic screens in many organisms, TILLING promises to be generally applicable. TILLING is no different from traditional mutation breeding as far as the organism is concerned, so genetically modified organism issues do not arise. This makes TILLING an attractive strategy not only for functional genomics, but also for agricultural applications.

The impetus for TILLING arose from a graduate student's frustration with the limitations of reverse-genetic methods available for Arabidopsis in the late 1990s. The original TILLING method used a commercial denaturing HPLC (DHPLC) apparatus for mutation discovery. However, this method would not scale up easily, and so alternative technologies are required. A method for enzymatic mismatch cleavage described by Tony Yeung seemed particularly attractive (Oleykowski *et al*., 1998). The LI-COR gel analyzer system (Lincoln, NE) (Middendorf *et al*., 1992) is ideally suited for this application. By mid-2001, robust protocols and software to begin a TILLING production operation with mutagenized *Arabidopsis* populations have been established (Colbert *et al*., 2001).

For TILLING *Arabidopsis thaliana* (Fig. 1), seeds were mutagenized by treatment with ethylmethanesulfonate (EMS). The resulting M_1 plants are self-fertilized, and M_2 individuals are used to prepare DNA samples for mutational screening, while their seeds are inventoried and sent to the Arabidopsis Biological Resource Center (ABRC) for eventual distribution. The DNA samples are pooled and arrayed in microtiter plates, and the pools are amplified using gene-specific primers. Amplification products are incubated with the CEL I endonuclease, a member of the S1 nuclease family of single strand-specific nucleases (Oleykowski *et al*., 1998). CEL I cleave to the 3' side of mismatches and loop outs in heteroduplexes between wild-type and mutant DNA while leaving duplexes intact. Cleavage products are electrophoresed using the LI-COR gel analyzer system, and a standard commercial image processing program is used to examine the gel readout. Differential double-end labeling of amplification products allows for rapid visual confirmation because mutations are detected on complementary strands and so can be easily distinguished from amplification artifacts. Upon detection of a mutation in a pool, the individual DNA samples are similarly screened to identify the individual carrying the mutation. This rapid screening procedure determines the location of a mutation to within ±10 bp for PCR products that are 1-kb in size. For the current mutagenized *Arabidopsis*

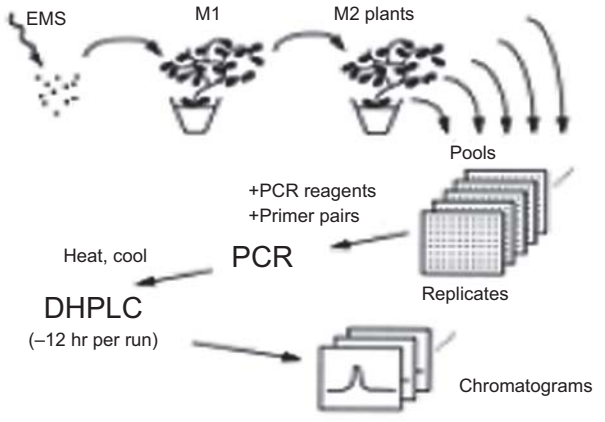

Fig. 1: Schematic depicting the TILLING strategy applied to *Arabidopsis*

populations with a density of 1 mutation per 235 kb, or approximately 4 point mutations per 8-fold pool gel representing 768 plants (Greene *et al*., 2003).

A key advantage of high-throughput TILLING over competing methods is that the approximate position of each detected mutation is inferred from the size of the fragment, which greatly facilitates subsequent sequencing. Furthermore, the double-end labeling strategy provides confirmation within the pool screen, and further confirmation comes from identifying the same fragments in tracking down individuals. Therefore, sequencing is done with near certainty that a mutation exists within a small interval. Examination of a sequencing gel trace in the predicted location suffices to identify the mutated base and the substitution, and we use Sequencher trace analysis software (Gene Codes, Ann Arbor, MI) to facilitate this step. Finally >3,000 *Arabidopsis* mutations were identified by this procedure, typically using the readout from only the strand in which the primer is closer to the detected mutation. By contrast, methods that do not provide an approximate location for a detected mutation, such as DHPLC, require that the full amplified segment be interrogated by sequencing, and for a 1-kb segment this would require multiple runs to be carefully scrutinized. Detection of heterozygotes under such circumstances can be challenging, especially when peak heights vary, and false positives will greatly exacerbate this problem. The increasing use of such mutations in evolution, crop improvement using conventional induced mutagenesis and high throughput screening (TILLING) will highly be appreciated among the breeders and biotechnologists in 21st century.

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