Chapter

Genetic Variability through Induced Mutation

Faisal Saeed Awan, Bushra Sadia, Javaria Altaf, Madiha Habib, Kiran Hameed and Shabbir Hussain

Abstract

The success of plant breeding is based on the accessibility of genetic variation, information about desired traits with well-organized approach that make it likely to develop existing genetic resources. Food security demands to break the yield barrier through increasing new cultivars which can adapt to wide range of environment. It is especially important to observe the character association for yield along with its components before recognizing novel technique to break the yield barrier. There are numerous methods for improved exploiting of the inherent genetic makeup of crops with heritable variations. It is recommended that recognized parental resources can also be induced to mutate for unmasking novel alleles of genes that organize the traits suitable for the crop varieties of the 21st century world. Chemical mutagens have extensively been applied to make genetic changes in crop plants for breeding investigation as well as genetic studies. Ethyl methane sulphonate (EMS) is the most frequently applied as chemical agents in plants. EMS normally induces GC → AT transitions in the genome causing mutated protein that performed different functions rather than normal. It is exposed that the utilization of EMS is an efficient approach for developing novel gene pool.

Keywords: ethyl methane sulphonate, mutation, genetic variability, singe nucleotide polymorphism

1. Introduction

Plant breeding involved in rapid introduction of genetic variability in plants, to divulge them with desirable characteristics, through genetic mutation. Plant evolution and genetic breeding depends on the genetic variation that, not all come from spontaneous mutation rather it comes by genetic recombination within population and their interactions with environmental factors [1]. Conventional breeding depends on prevailed environmental genetic variations in wild and cultivated plants that require a large-scale backcrossing to incorporate it and stabilize it while, new mutation breeding strategy is less time consuming and easy that enhances the selection of desirable mutants [2]. Mutation breeding is an advancement of plant breeding where the induction of physical and chemical mutagens cause genetic variation. These variations are transferred to next generation through recombinant hybridization in meiosis [3]. Selection of breeding individuals only probable when there is a significant genetic variability exists [4]. Mutation breeding depends upon the transfer and stabilization of heritable characters that cause the variability [5].

Spontaneous and Induced mutations are the primary source of all variations exists in an organisms that may be plants or animals [6]. Genetic variability endorses the differences among the same species and its existence in population is essential for its survival with changing environment. Induced mutation with specific mutagens generates the individuals with desirable characters that can be further exploit in breeding to generate new verities in plants [7, 8]. Mutation was first recognized in the late nineteenth century by Hugo de Varies, when he was working on the 'rediscovery' of Mendel's laws of inheritance [6]. Chemical mutagens are less harmful and easily available for work. In plants, widely used chemical mutagen is EMS that is very effective in causing point mutation in genome [9]. Mutation approaches produces huge and minute effect on all types of phenotypic traits [6]. Induced mutation is helpful in growing novel cultivars of plants as seedless grapes and edible bananas [3, 10] also it bring out the novel color variants of tuber and root crops [11]. Mutagenesis also apply to improve dwarfness, early growth, resistance to biotic and abiotic stresses, and yield improvement as well as quality enhancements in plants [12–15].

Novel breeding techniques based on biological mutagenic agent are widely introduce in plants for targeted variation is also known as targeted genome editing [16]. These genome editing techniques cause the specific and precise genome mutations. It introduce targeted mutation by either insertion, deletion that disturbs the function of gene. CRISPER/Cas9 is a novel technique that introduce desired targeted mutation permanently inside the genome [17].

2. Brief description of induced mutation

Mutation breeding carried out through three types of mutation as induced mutation, site-directed mutation and insertion mutation [1]. Induced mutation is a tool of generating variability artificially [18]. Mutagenesis is sudden and heritable changes in genetic sequence that stimulated by some mutagens like physical, chemical as well as biological agents [19]. Mutagenesis became well-known in 1950s when various crop species were largely induced through irradiation to enhance trait divergence [20]. Natural mutants are typical type of spontaneous mutations that generate modern phenotypes without human beings interference such as seed dispersal, thin seed coat, seed dormancy and reduced seed length. Heritable mutants are appropriate for human utilization for example loss of bitterness in various types of nuts, almonds, watermelons, potatoes, lima beans, egg plants as well as cabbages. Dwarfing genes exploited to increase grain yield in 1960s. It was completed with the introgression of natural mutant alleles into rice as well as wheat genome. The main disadvantage of this mutation is the loss of numerous wild features in crops [21].

Physical and chemical mutations are collectively called as induced mutation. Induced mutations have a record of 83 years as the first reported [22, 23] in plants. In 1927, Muller illustrated that X-ray induction could enhance the mutation rate in a *Drosophila* up to 15,000% [22] then Stadler examined a powerful phenotypic divergence in barley and maize by induction of X-rays and radium [23]. Induced mutations in plants originated directly from X-rays, radioactivity as well as radioactive elements through Roentgen (1895), Becquerel (1896) as well as Marie with Pierre Curie (1898) respectively. The Nobel Prize was awarded to Roentgen, Becquerel, Marie and Pierre Curie for successful mutation induction [24]. Nitrogen mustard is composed of poisonous mustard gas that applied in World Wars I and II. It is a chemical mutant that exposed to introduce mutations in cells [25]. Agents that cause artificial mutation are called mutagens that includes physical mutagens (X-ray, Gamma rays, Neutrons, Alpha/beta particles) and chemical mutagens (Alkylating agents, Azide, Hydroxylamine, Antibiotics, Nitrous acid) [1].

Replace the entirety of this text with the main body of your chapter. The body is where the author explains experiments, presents and interprets data of one's research. Authors are free to decide how the main body will be structured. However, you are required to have at least one heading. Please ensure that either British or American English is used consistently in your chapter.

2.1 Physical mutagens

Radiation is described as energy transfer in the sort of particles and waves [26]. These radiations are types of the electromagnetic (EM) spectrum that generates ions so it is also called as ionizing radiation. Ionizing radiations are the most frequently used physical mutagens [27]. Approximately, seventy percent of mutant varieties were generated by action of ionizing radiations in past eighty years [27]. These radiations consist of cosmic, gamma (γ) as well as X-rays [28]. Practical mutations by cosmic radiation have been reported in rice, cotton, wheat, tomato, sesame and pepper [29] as well as in maize [30]. The most universally applied physical mutagens are gamma and X-rays [27]. X-rays were the primary mutagens that applied to stimulate mutations [26]. However, gamma rays have gained popularity when these rays were accessible by the in several developing countries [31]. Gamma rays are less harmful produce point mutations with minute deletions while, fast neutron produces chromosome losses, translocations with huge deletions [32]. Additional physical agents are subatomic particles known as alpha (α), beta (β) particles, neutrons as well as protons. These particles are ionizing agents [26]. Ultraviolet (UV) rays are non-ionizing. These rays have potential of tissue penetrability for mutagenesis. Recently, plant materials have been thrown out into space for analysis of mutagenesis. Nevertheless, information about genetics of space induction is so far insufficient [1].

2.2 Chemical mutagens

Researchers search for another source for producing mutations due to the high chromosomal irregularity from ionizing emission. Consequently, a group of chemical induction has been exposed [33]. There are some chemical mutagens namely alkylating agents, base analogues as well as intercalating agents [25]. Alkylating agents were the primary group of chemical mutagens to be exposed by Auerbach and Robson [34] when they discovered the mutagenic result in mustard gas throughout World War II. Chemical mutagens consist of nitrogen mustards, sulfur mustards, ethyl methane sulphontes, ethyleneimines, epoxides, alkyl methane sulphonates, ethyleneimides, alky lnitrosoamines and alkyl nitrosoureas [35]. Chemical mutagens are more applicable for introduction of in-vitro mutation as compared to radiation approaches [36]. Chemical mutagens introduces single base pair (SNPs) change as compared to translocations and deletions as occurred in physical induction that induce more damage with harshly decrease viability. They are simple to apply rather than physical agents [32]. However, undesirable changes are usually high in chemical induction as compared to physical induction [26]. However, these mutagens are usually carcinogenic. Mustard gas, ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS) as well as nitrosoguanidine are powerfully carcinogenic that should be used carefully [37].

2.2.1 Introduction and mode of action of ethyl methane sulfonate (EMS)

Ethyl methane sulfonate is the most frequently applied in plants among chemical mutagens [37] due to its efficiency and accessibility [38]. It has capability to generate the high and stable nucleotide substitution in diverse genomes of organisms [39, 40].

This chemical generates a huge quantity of point mutations in relatively little mutant population. This chemical is enough to develop the genome mutations [40]. EMS has major role in forward genetic for screening of various organisms. It is also applied in model animal and plant for mutagenesis named *Drosophila melanogaster* as well as *Arabidopsis thaliana* respectively. EMS is extraordinarily reliable due to similar levels of induction have been attained in model organisms for example base replacement are analogous for *Arabidopsis* seeds immersed in EMS [41, 42] as well as EMS-fed males *Drosophila* [43]. EMS causes suitable levels of lethality as well as sterility [40]. Genome size does not show to be a significant issue in EMS mutagenesis. Nevertheless, EMS toxicity may differ from species to species [44]. It is also applied in high throughput selection such as TILLING populations [37] in plants.

Ethyl methane sulfonate forms an abnormal base of O^{-6}-ethyl guanine due to alkylation of guanine bases. During DNA duplication, it located a thymine residue above a cytosine residue result in an accidental point mutation. Approximately 70–99% alterations in EMS-treated populations are due to GC → AT base pair conversion [37, 40].

2.2.2 Dose of mutagen

LD_{50} is the percentage of test material that are killed by a specific dosage of chemical or radiation mutagen in which half test material will be die. Fixation of LD_{50} is important before the start of an experiment in induced mutation. These doses vary according to fluctuation in treatments duration, quantity, pH as well as solvent used. Mutagen dose can be caused low or high mutation frequency as a result of ignoring the importance of LD_{50} [45–47]. Doses lower than LD_{50} favor plant's recovery after treatment, while the use of high doses increases the probability to induce mutation either in positive or in negative direction. The efficiency of mutation is determined by concluding the accurate doses of mutagens if the dose is random it creates higher number of harmful mutations in each plant [48]. The mutation quality of practical mutation is not absolutely correlated to dose rate. High mutagen doses did not produced the excellent results of yield [26]. Seeds of *Oryza sativa* L. spp. *Indica* cv. MR219 were mutagenized to different doses of EMS from 0.25–2%. Seeds were incubated for ten to twenty hours for establishment of kill curve as well as sensitivity of the tested genotypes [49].

2.3 Mutagenesis with biological agents

Insertion mutageneis with biological agents involved in insertion or deletion of some sequence in genome. It may cause random mutation at genome as transfer DNA, retro-transposon and transposon. Also the insertion mutations can be site specific or targeted that cause genomic variation at specific site included the novel genome editing techniques.

Transfer DNA (T-DNA) insertion mutation helps in identification of gene function in plant genome. This insertion mutagenesis cause loss or gain of gene function that can be observed by phenotypic response [50]. It can also identified through whole genome sequencing or using the *Agrobacterium tumefaciens* machinery that insert the T-DNA at specific flanking sites.

2.3.1 Mutation by genome editing tools

Plant breeding relies on incorporation of genetic variation for desired traits. The innovative strategies are exploited from many years to reduce the off-target random mutations caused by physical and chemical mutagens [16]. These technologies

includes: site-directed nucleases, RNA-dependent methylation, oligonucleotide directed mutagenesis, agro infiltration, cisgenec/intragenic and reverse genetics. Site-directed nucleases including Zinc finger nucleases (ZFN) [51], transcription activator-like effector nucleases (TALEN) [52] and Clustered regularly interspaced short palindromic repeats (CRISPER) and CRISPER-associated nuclease 9 (CRISPER/Cas9) [53] system had revolutionized the mutation breeding strategy by introducing targeted genome editing. TALEN and CRISPER/Cas techniques precisely and permanently incorporate the desired DNA into the genome and hence cause genetic variation [54].

CRISPR system is a well settle defense system that generate acquired immune response for resistance to bacteria, fungi and phages [55]. It was originated from bacteria and consist of repetitive DNA genetic codes as well as proto-spacer DNA (defensive genetic codes formed during exposure to pathogens) [56, 57]. Cas9 is an endonuclease that generate double stranded break in DNA through its two active domains [58]. Cas9 creates the break at targeted site by utilizing the guided RNA sequence. The CRISPER/Cas system recognized the specific site at genome through guide RNA (gRNA) [59] and at targeted site Cas9 creates a double stranded break (DSB). These break are repaired by DNA repairing system that ultimately cause mutation by either non-homologous end joining (NHEJ) or homologous recombination system [HR] [60, 61]. Specific base-pair change occur during DNA repair system as Cytosine to thymine (C/T) [62] and Adenine to guanine (A/G) [63] that observed in several crop plants as canola, rice, tomato, wheat and corn [57]. CRISPR/Cas9 system was used in rice to generate semi-dwarf mutants in rice from T2 to T4 generation. Stable indels passed through generation producing homozygous mutant [64]. In plants, CRISPR/Cas system generates induced mutation through gene knockouts, insertion or generating single nucleotide polymorphism (SNP) in plants [56, 57]. Some latest gene editing mutations using CRISPR machinery enlisted in **Table 1**.

Gene targeted	Vector: promoter	Transformation method: promoter	Plant variety	Mutation nature	Reference
BnaMAX1s	Gateway 100 vectors; BGK01 vector: 35S promoter	Agrobacterium GV3101	Rapeseed RS862	Knockout mutation	[65]
GmFT2a; G,FT5a	pTF101.1: 35S promoter	Agrobacterium strain EHA101	Soybean jack variety (*Glycine max*)	Knockout by CRISPR	[66]
OsRR22	pYLCRISPR/ Vas9Pubi-H; Cas9-OsRR22-gRNA: OsU6 promoter	Agrobacterium EHA105: OsU6	Rice japonica WPB106	Knockout mutation	[67]
MaGA20ox2	pYLCRISPR/ Cas9P_{ubi}-H vector: U3 promoter	Agrobacterium strain EHA105	Banana cultivar Gro Michel) (Musa acuminate)	Mutation as insertion and deletion	[68]
Exon of *SD1* gene	pBIN-sgR-Cas9-OsU3 vector: 35S PROMOTER	Agrobacterium strain LBA4404, EHA105	Rice variety 9815B, JIAODA138, HUAIDAO1055	On target and off target mutations	[64]
BnSFAR4; BnSFAR5	pCas9-TPC: pMP90RK	Agrobacterium GV3101	Rapeseed RS306	Knockout mutation	[69]

Gene targeted	Vector: promoter	Transformation method: promoter	Plant variety	Mutation nature	Reference
63 immunity associated genes	P201N-Cas9: U6 promoter	Agrobacterium ID1249 strain	Tomato RG-PtoR or RG-prf3	Short Indels	[70]
VvMLO3; VvMLO4	pYLCRIPSR/Cas9-N vector: *AtU3b* and *AtU6–1* promoter	Agrobacterium strain GV3101	Grape wine PN40024 (*Vitis vinifera*)	Short Indels	[71]
HvITPK1	pYLsgRNA-OsU6 pYLCRISPR/ Cas9Pubi-H: U6 promoter	Agrobacterium strain AGL1	Barley model cultivar Golden Promise	Insertion mutation	[72]
Clpsk1	pRGEBB320cas9-gRNA-Clpk1: AtU6 promoter	Agrobacterium strain EHA105	Watermelon Sumi 1	Lnockout	[73]
GhCLA and GhPEBP	pRGEB32-GhU6.7: cotton U6 promoter	Agrobacterium strain GV3101	Cotton Jin668 (*Gossypium hirsutum*)	Point mutation	[74]

Table 1.
Induced mutation in plants using targeted genome editing method CRISPR/Cas9.

3. Identification of mutagenic site through molecular marker

Markers have been used for cultivar recognition as the first light of forward genetics. Markers fall into three major categories as visually measurement of traits, gene product as well as DNA test known as morphological markers, biochemical markers as well as molecular markers respectively [75]. Classical breeding can be fast-tracked using molecular marker approaches for identification of mutagenic regions and to access the variations inside genome [76]. These markers lies inside the genetic region or nearby it. Newest genotyping approaches like genotyping by sequencing (GBS) made it easier to identify even a single base pair change as single nucleotide polymorphism (SNP).

3.1 Morphological markers

Most of the induced mutants have been released as cultivars by selection through morphological markers. These markers based upon agronomic traits such as maturity, height, early flowering, fruit appearance, seed quality as well as resistance to diseases that can be monitored easily as a result of their epiphytotic character. Huge morphological, physiological as well as ecological differences has been existed in cultivated rice genomes. It is a general approach employed to determine genotypic relationship [77].

Detection of morphological traits is performed by statistically method. Multivariate methods have statistical approaches that widely used in telling the intrinsic variation among various crop genotypes. Multivariate analysis has been reported for study of genetic diversity in numerous crops such as barley [78], sorghum [79], wheat [80], peanut [81] and rice [82].

3.2 Genetic markers

Mutant phenotypes were usually recognized depend upon their morphological characteristic. But morphological markers are not steady due to less heritability

along with complex genetic nature [83]. Then novel method have been developed depend upon genetic information of DNA. This procedure is a faster and more reliable as compared to other methods. Molecular markers depend upon genetic variant in the genome [84]. DNA-based markers have basically replaced previous biochemical markers as importance of DNA analysis has been reported in plants [85]. DNA markers are largely scattered across the entire genome due to larger in quantity [75]. Genetic markers perform main role for reorganization of heritable traits in plant breeding as well as genetics [86].

3.2.1 Types of genetic marker

Numerous types of molecular markers have been utilized. Molecular markers include restriction fragment length polymorphism (RFLP) [87], random amplified polymorphic DNA (RAPD) [88], amplified fragment length polymorphism (AFLP) [89], inter-simple sequence repeat (ISSRs) [90], microsatellite or simple sequence repeats (SSRs) [91] and single nucleotide polymorphisms (SNPs) [92] are currently accessible to evaluate the diversity and variability at the DNA level.

3.2.1.1 SSRs as a sequence based marker

These markers are group of tedious DNA chain typically two to six base pairs. It is a form of VNTRs (Variable Number Tandem Repeats) [93]. These markers are well-known as STRs (short tandem repeats). The rate of different STRs length is feature of microsatellite loci in rice [75]. They consist of dinucleotide; trinucleotide as well as tetranucleotide repeats for genetic analysis. Dinucleotides are the key form present in most vertebrates. Trinucleotide repeat are rich in plants [94]. Microsatellite markers are believed to be suitable over different array of markers due to following reasons. These are scattered all over the genome of extremely conserved region. These markers have various qualities of simplicity, high polymorphism, rapidity as well as stability. These markers have been model for examination of germplasm, genetic diversity [95], heterosis, purity test, gene mapping, fingerprints assembly, phylogenetic comparison as well as marker aided selection [75, 95]. A random collection of SSRs assist in estimation of rice genetic diversity and rice cultivar classification without mistakes [96]. Particularly SSRs markers have been extensively employed in rice genetic analysis for high allelic detection [97]. Microsatellite exposed unreliable level of genetic relationship among the domesticated as well as wild collection of rice [98].

3.2.1.2 SNPs as genetic markers from high-throughput sequencing

SNPs (single nucleotide polymorphisms) signify a strong group of genetic markers [99] among different categories of molecular markers due to following reason [100]. These markers detect single-base pair location depend upon sequence variation in genomes [101]. SNPs markers offer a huge marker density in genomes [102]. SNPs markers have achieved significant importance in plant genetic analysis due to their brilliant genetic qualities, genetic diversity, evolutionary interaction [103], high throughput genetic mapping [104], population substructure [105], genome-wide linkage disequilibrium [106] as well as association mapping [107]. Availability of high quality reference genome sequence made it easier to scan out mutation by re-sequencing the species genotypes through next generation sequencing (NGS) approaches and to identify the variation in targeted genotype through mapping techniques as genome-wide association mapping [108].

Classically, a quite large sequencing attempt is faithful to recognize polymorphic location in a genome among a set of various breeding lines [109]. A precise

multiplexed SNPs genotyping analysis is necessary to utilize the huge SNPs source for high-throughput genetic test in rice [110]. It will become routine to re-sequence the plants genome with current SNPs platforms as the price of genome sequencing keep on to reduce [111].

SNPs genotyping have been applied in many organism including rice [112–114], *Arabidopsis* [115], maize [116], soybean [117] and wheat [118]. The high class order of the rice genome has offered genome-wide SNPs source [119]. Polymorphic loci (5.41 million) were detected between the two main domesticated rice subspecies (*Indica* and *Japonica*) by SNPs genotyping [120].

A complete map of rice genome builds 6,119,311 SNPs variants for 1529 genome orders. SNPs (213,188) were located in *Indica* and *Japonica* rice. Asian and African rice were established 9595 SNPs [121]. Three thousand rice genomes project [122] submit for rice clustering of aus/boro genotypes. Only 208 accessions are categorized as aus/boro depends upon SNPs markers of 200,000. It is also exposed from further study that aus group was genetically related by 376,000 SNPs markers [123].

4. Conclusions

The considerable amount of phenotypic variability can be identified by employing highly sophisticated Molecular approaches like SSR and SNPs within the mutant populations. These genetic changes indicated that EMS might be helpful for the development of desired genetic changes in crop plants. It was also recommended that current SSRs and SNPs markers could be suited in further analysis for estimation of genetic diversity of rice mutants.

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Conflict of interest

"The authors declare no conflict of interest."

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Author details

Faisal Saeed Awan[1*], Bushra Sadia[1], Javaria Altaf[2], Madiha Habib[1], Kiran Hameed[1] and Shabbir Hussain[1]

1 Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture Faisalabad, Pakistan

2 Department of Zoology, Government College University Faisalabad, Pakistan

*Address all correspondence to: faisal.saeed@uaf.edu.pk

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