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Chapter

Current Strategies and Future of Mutation Breeding in Soybean Improvement

Alp Ayan, Sinan Meriç, Tamer Gümüş and Çimen Atak

Abstract

Soybean, which has many foods, feed, and industrial raw material products, has relatively limited genetic diversity due to the domestication practices which mainly focused on higher yield for many centuries. Besides, cleistogamy in soybean plant reduces genetic variations even further. Improving genetic variation in soybean is crucial for breeding applications to improve traits such as higher yield, early maturity, herbicide, and pest resistance, lodging and shattering resistance, seed quality and composition, abiotic stress tolerance and more. In the 21st century, there are numerous alternatives from conventional breeding to biotechnological approaches. Among these, mutation breeding is still a major method to produce new alleles and desired traits within the crop genomes. Physical and chemical mutagen protocols are still improving and mutation breeding proves its value to be fast, flexible, and viable in crop sciences. In the verge of revolutionary genome editing era, induced mutagenesis passed important cross-roads successfully with the help of emerging supportive NGS based-methods and non-destructive screening approaches that reduce the time-consuming labor-intensive selection practices of mutation breeding. Induced mutagenesis will retain its place in crop science in the next decades, especially for plants such as soybean for which cross breeding is limited or not applicable.

Keywords: soybean, mutation breeding, mutagens, induced mutagenesis, next generation sequencing

1. Introduction

Soybean (*Glycine max* (L.) Merrill.) has a central position in agriculture along with barley, cassava, groundnut, maize, millet, potato, oil palm, rapeseed, rice, rye, sorghum, sugar beet, sugarcane, sunflower, and wheat which were considered as the most cultivated plants worldwide. Its central role is not only constituted due to the dense protein and high-quality oil contents but also industrial raw material supply. Tofu, soy milk, soy sauce, and miso are the main nutritious human soy products. Also, extracted soy oil, with over 75% oleic acid and under 10% polyunsaturated fatty acids, is one of the most preferred oils sold commercially in the United States today [1]. Long shelf-life required fry, spray, and ingredient oils should preferably contain higher oleic acid due to the better persistence to oxidation. Soy meal is also a major

source of protein used in pig and poultry industries. The companion animal industry prefers soy meal as a protein source in animal diet, especially for dogs. High-quality amino acid composition and highly digestible protein content leads to the use of soy meal in aquaculture diets [2]. On the other hand, soy oil has various industrial uses as pharmaceuticals, plastics, papers, inks, paints, varnishes, and cosmetics.

In the verge of global warming effects, renewable energy sources as an alternative to fossil fuel are getting importance. Soybean is also an important biodiesel crop in many countries along with maize, especially in South America countries [3]. Besides the alternative bioenergy crop role, it has also environmental effects as being capable of utilizing atmospheric nitrogen through biological nitrogen fixation and is therefore less dependent on synthetic nitrogen fertilizers. While drought is one of the most plant growth and development limiting factors in present days, nitrogen deficiency is equally crippling for plants, as well, due to its structural, genetic, and metabolic functions in crop yield. Highly stable and non-reactive N_2 is the most abundant constituent of the Earth's atmosphere, still no eukaryotic organism can use it directly. Some members of *Leguminosae* (*Fabaceae*) family including soybean have adopted the ability to establish symbiotic interactions with diazotrophic bacteria known as rhizobia in evolutionary adaptations. By this means, a process called 'biological nitrogen fixation is a low-cost N source that sufficiently increases soybean yield with low environmental impact and avoids the use of synthetic N fertilizers [4].

Soybean (*G. max* (L.) Merr) as a member of the family *Fabaceae*/Leguminosae, subfamily *Papilionoideae,* and the tribe *Phaseoleae* contains two subgenera as *Glycine* which has 26 perennial species and *Soja* (Moench) F.J. Herm. having four annual species [5]. Domestication of cultivated soybean can be traced back to China in 5000 years ago, however, the geographical origin of *Glycine* genus can be traced back to putative ancestor (2n = 2x = 20) which was presumably migrated and formed unknown or extinct wild perennials (2n = 4x = 40) in China. Wild annuals (2n = 4x = 40; *Glycine soja*) and domesticated soybean (2n = 4x = 40; *G. max*) subsequently evolved [6]. The genetic diversity of *G. max* is assumed to regress due to man-made genetic bottlenecks through selection for high yielding lines in modern plant breeding applications. Indeed, yield is the backbone of the profitability and the feasibility. Varieties with other superior traits are not significant in industrial scale unless they have a high yield. As well as yield, maturity, herbicide, and pest resistance, lodging resistance, shattering resistance, seed quality and composition, abiotic stress tolerance are other breeding selection targets [7]. While the wild relative *G. soja* grows in various environmental conditions and have not been exposed to the selective bottlenecks, it retained significant genetic diversity over time.

On the other hand, soybean flowers represent cleistogamous characteristics. Cleistogamy, which is described as the production of both open (chasmogamous, CH) and closed (cleistogamous, CL) floral forms by one species, is very common among angiosperms. Soybean is pseudocleistogamous cleistogamy in which no morphological differences between CL and CH flowers occur other than a lack of expansion of petals and anthesis in CL flowers. It may also be induced by environmental stress factors, occasionally. Cleistogamy is observed both in cultivated soybean [*G. max* (L.) Merr.] and its wild relative [*G. soja* Sieb. & Zucc.]. Soybean usually produces both CH and CL flowers on the same plant. In these plants, fertilization occurs within closed petals of CL flowers [8–10]. The rates of natural cross-pollination have been observed between ranges of 0.03–1.14% in natural conditions for self-pollinating soybean plant [11]. Thus, cleistogamy may have influenced the genomic homogeneity and reduced genomic variation further in soybean along with domestication practices.

In this context, improving genomic variations is crucial in soybean breeding. This chapter will summarize present conventional and biotechnological methods in soybean breeding and emphasize on mutation breeding practices with the concluding discussion on future prospective.

2. Improving genomic variations

2.1 Conventional methods

In soybean breeding, oil and protein content, resistance to biotic and abiotic stresses have been the main breeding objectives in past decades. In conventional breeding practices variability of desired traits is based on the detection of novel genotypes which contains enhanced characteristic for the trait. Hybridization of these novel genotypes with the varieties which are already in use for commercial production is the base of the process. Subsequent, the selfing of progenies, which contain traits distributed according to basic genetical segregation rules, provide novel genotypes. Detection of the most favorable recombination in those progenies which is also referred as homozygosity by selection is based on numerous selection methods including pedigree selection, single-seed descent, bulk breeding, mass selection, selection among half-sib families, selection within half-sib families. However, the traditional pedigree method and the single-seed descent method (SSD) are the most successful and preferred in soybean breeding. The last step in the process is yield testing. Available genotypes and technical infrastructure (agricultural machines, greenhouses, and experienced stuff) as well as breeding objective are deciding factors in method selection. Breeding objectives generally depend on the local agroecological conditions, available acreage, production intensity, market demand, and economical value [11–13].

Pedigree selection is a highly labor-intensive method that depends on visual selection by the appearance in each generation. In this method, desirable genotypes are selected in each generation and the limited number of selected genotypes are advanced to the next generation by inbreeding/selfing. The labor intensity of the method is limiting for large scale breeding practices [14]. Single-seed descent (SSD) is the most preferred method with pedigree selection to increase homozygosity in soybean. Single pod descent (SPD) accelerates the SSD for harvesting process even further. This method is mostly preferred for high seed yield, oil content and quality, resistance to biotic and abiotic stresses and maturity duration breeding objectives [15].

2.2 Biotechnological approaches

Although, the improvement of plants by conventional breeding methods is one of the most preferred breeding strategies, the limited hybridization among species, transfer of undesirable genomic segments together with genes of interest (e.g., linkage drag) and the fact that diversity in species is based on spontaneous mutations with a very low frequency necessitated the development of new breeding strategies. Plant breeding has often benefited from new technologies to overcome such limitations. Molecular breeding as one of these strategies can be extensively defined as the utilization of genetic manipulation of DNA at the molecular level to improve of trait of interest in plants, including genetic engineering, molecular marker-assisted

selection, marker-assisted backcrossing, marker-assisted recurrent selection, genome wide selection [16, 17]. Molecular breeding requires more complex equipment and molecular tools compared to conventional breeding approaches. The identification of functional genes and DNA markers associated with variation at the genomic level is an important part of molecular breeding. Marker-assisted breeding (MAB) which utilized marker-assisted selection involves the use of molecular markers in conjunction with linkage maps and genomics, and the improvement of crop plant traits based on genotypic analyses. Moreover, MAB requires minimum phenotypic information during the training phase. The convenience of use and analysis, low cost, a small amount of DNA requirement, co-dominance, reproducibility, high-rate polymorphism and genome-wide distribution are the most important factors for molecular tools used in marker-assisted breeding (MAB) in plants [18]. Along with the emergence of marker-assisted selection (MAS) after the mid-1980s, rapid improvement of plant yield and quality has been achieved thanks to the development of molecular maps by utilizing structural and functional genomics in plant breeding. MAS can be classified into five broad areas: marker-assisted evaluation of breeding material; marker-assisted backcrossing; marker-assisted pyramiding; early generation selection and combined MAS [19].

DNA markers have made significant contributions to increasing the efficiency of conventional and mutation breeding through marker-assisted selection and have been integrated into traditional schemes to develop novel varieties or used instead of traditional phenotypic selection. Many DNA marker techniques have been developed based on different polymorphism detection techniques or methods (such as nucleic acid hybridization, restriction enzyme digestion, PCR, DNA sequencing) such as RFLP, AFLP, RAPD, SSR, SNP. Advances in molecular marker techniques and the creation of large-scale marker datasets provide a reliable way to identify and trace the genetic basis of important agricultural traits. Molecular markers developed from functional genes have been used for the development of soybean varieties by improving important agricultural traits such as yield, disease resistance and abiotic stress tolerance [20]. Breeders can combine all the suitable alleles in a single variety to develop desired crops, thanks to molecular markers closely related to particular traits. However, although soybean yield remains the most important selection criterion for soybean breeders and the primary factor for profitability, it is very difficult to acquire complex traits such as yield, quality and abiotic stresses with marker-assisted selection. Genomic selection (GS) is a promising approach that leverages molecular genetic markers to design new breeding programs and develop new marker-based models for genetic evaluation. GS, which has high selection accuracy, reduced selection duration, greater gain per unit time, precise and accurate results provide breeders with opportunity faster development of improved crop varieties for complex traits. New marker technologies, such as NGS-based genotyping, have made the use of genomic selection as routine for crop improvement while increasing the efficiency of marker applications. The availability of genome-wide high-throughput, low-cost and flexible markers, usability for crop species with or without a reference genome sequence with a large population size are the most important factors for its successful and effective implementation in crop species [21].

Plant breeders have begun to take advantage of molecular breeding more through advances in the identification of QTLs/genes responsible for important agronomic traits. Numerous quantitative character loci (QTL) mapping studies performed for a variety of agricultural crops have resulted in the association of DNA markers and traits. The most notable high-throughput genotyping system is single-nucleotide

polymorphisms (SNPs), which are heavily used in quantitative character locus (QTL) discovery. More than 10,000 QTLs using different marker systems have been reported in more than 120 studies involved 12 plant species aimed at improving quantitative properties with economic importance [22]. Linkage analysis for QTL mapping is frequently preferred in two-parent populations. Genotyping by next-generation sequencing become prominent as a promising technology and is also used for genome-wide association studies (GWAS) to identify useful genes to increase crop productivity. Soybean genome sequence information, as one of the most substantial resources, is the basis of genomic studies and has allowed the significant development of genomic applications for soybean breeding.

As in transgenesis, studies involving the transfer of a limited number of loci from one genetic background to another are also within the scope of molecular breeding. Especially in the last two decades, genetic engineering approaches that generate novel genetic variations in plant genome or enable the transfer of gene of interest for obtaining original traits to plants have been frequently preferred among the biotechnological approaches that have been successfully applied in plant breeding [23–25]. Along with recent developments in recombinant DNA technology, it has been paved the way for transferring the desired characteristics to plants within plant breeding in a short time. These genetic engineering and plant transformation approaches which make plant breeding faster, more predictable and improvable for a wide variety of species, include successful characterization, cloning, modification and transfer of DNA expressed the desired trait into cells. The gene pool utilized by plant breeders in conventional breeding since the mid-1990s has been considerably expanded by genetic transformation approaches and many different transgenic plants have been developed by transferring traits that are tough to transfer [26–28]. Genetically Modified Organisms (GMOs), whose agricultural traits have been improved through inter-species gene transfer by utilizing genetic engineering techniques, have been increasingly planted, globally. The total cultivation areas of approved GM plants have increased approximately 113 times, from 1.7 million hectares in 1996 to 191.7 million hectares in 2018. This increase reveals that transgenic technology is the fastest adopted technology in recent years. A total of 2.5 billion hectares or 6.3 billion acres GM crops have been planted in the first 23 years (1996–2018) of commercialization of transgenic plants [29]. Especially soybean (95.9 million hectares) which comprises 50% of the global area of GM crops, corn (58.9 million hectares), cotton (24.9 million hectares) and canola (10.1 million hectares) are the four main transgenic crops cultivated. Transgenic crops, which were initially developed for only producers/farmers on the purpose of agriculture such as insect resistance and herbicide tolerance, afterwards were developed for other traits such as disease resistance, abiotic stress tolerance, modified product quality for both the producers/farmers and consumers. Especially cultivation of stacked events which are GM crops with more than one genetic modification, gather momentum.

During the 23-year period from 1996 to 2018, herbicide tolerance has accounted for the majority of transgenic crops area planted. Only herbicide tolerance cultivation areas of transgenic crops have been gradually decreasing over the years with the increasing importance of stacked cultivars with multiple traits (e.g., both insect resistance and herbicide tolerance; IR/HT). In 2018, stacked (IR/HT) traits used in soybean, maize and cotton have accounted for 42% of the total transgenic acreage, up 4% annually. Traits such as herbicide tolerance, insect resistance, disease resistance, pollination control, modified crop quality, anti-allergy, delayed fruit softening, delayed ripening, enhancement of vitamin A content, modified alpha-amylase, modified amino acid,

modified oil/fatty acid, modified starch/carbohydrate, nicotine reduction, non-browning phenotype, phytase production, reduced acrylamide potential, reduced black spot bruising have been transferred to plants and many of these have been combined in various combinations [29]. Thanks to these features brought to agricultural plants, the product yield obtained from the cultivation areas increases significantly. Along with the acceleration of the transfer of the appropriate gene combinations to plants with high added value, products that can provide significant gains in the agricultural economy have been developed. In this process, about 30 different types of transgenic plants such as particularly G. max (soybean), Zea mays (corn), Gossypium hirsutum (cotton), Bassica napus (canola) and including fruits and vegetables such as Phaseolus vulgaris (bean), Prunus domestica (plum), Beta vulgaris (sugar beet), Solanum melongen (eggplant), Cucumis melo (melon), Carica papaya (papaya) have been approved [30]. Stacked traits such as Intacta™Roundup Ready™ 2 Pro, Enlist E3™ and Vistive Gold™ soybeans are favored by farmers for their cost-saving technologies. In 2018, the planting of crops with novel stacked traits in various combinations, including herbicide-tolerant and high-oleic acid soybean, herbicide-tolerant and salt-tolerant soybean varieties were approved. The global acreage of soybeans in 2018 was 123.5 million hectares, of which 78% (95.9 million hectares) were GM soybeans. GM soybeans have been planted on 95.9 million hectares, 50% of the global cultivated area for GM crops; USA (34.1 million hectares), Brazil (34.9 million hectares), Argentina (18.0 million hectares), Paraguay (3.35 million hectares), Canada (2.42 million hectares), Uruguay (1.26 million hectares), Bolivia (1.26 million hectares) and Southern Africa (694,000 hectares). In the USA, soybean is the second most important crop with a total cultivated area of 36.26 million hectares in 2018, with 94% GM. These GM soybeans contain herbicide-tolerant traits that control a variety of weed species depending on the genes deployed. Other features incorporated into HT soybeans include consumer properties such as high monounsaturated oleic acid and enriched omega-fatty acid. In Brazil which has the second-largest GM crop cultivation area with 51.3 million hectares in 2018, GM soybean was planted in an area of 34.86 million hectares. As for Argentina which was the third country to plant the most GM crops in 2018, 18 million hectares of soybeans were planted [29, 30].

2.3 Mutation breeding

Term of mutation was first introduced by de Vries as the sudden and unexpected emergence of hereditary alterations in defining traits apart from recombination in Mutation Theory Vol. I [31]. In 1920s, following Stadler's experiments on genetic effects of X-rays on maize, plant breeders started to use physical and chemical mutagens to induce heritable mutations in plants [32]. As a term, mutation breeding is introduced to the scientific world by Freisleben and Lein defined as the deliberate exposure of biological materials to mutagens for induction of mutation frequency exceeding the natural mutation frequency to develop new varieties [33].

Mutations that cause genetic variation among living organisms can be categorized under spontaneous and induced mutation terms. Spontaneous mutations, which occur in low frequency and accumulate for a long time, allow plants to adapt very distinct environments apart from their original habitat [34–36].

The spontaneous mutation may occur due to the exposure to physical (cosmic radiation, natural background radiation of earth), chemical (alkylating agents, base analogs, antibiotics) mutagens and biological factors (transposon activation) during the reproductive stage. Spontaneous mutation frequency is calculated as

10^{-6} in plants during DNA replication, repair, or genomic element activities [37]. In vitro and in vivo propagation processes may also trigger gene methylation and cause epigenetic alterations while transposon mobility may trigger somoclonal variation and increase spontaneous mutations. Loss or activation of gene through transposable elements (TEs) regulate many biological processes. There are various studies on somoclonal variation-based trait improvement in plants. However, low mutation frequency is a real draw back for considering this method as common breeding alternative [35, 37]. Mutations can also be induced through physical and chemical mutagens. The use of mutagens may induce 103-fold more mutants comparing to the spontaneous mutations. Ossowski et al. [38] calculated spontaneous mutation frequency as $7x10^{-9}$ substitutions per site per generation for *Arabidospsis* plant in 30 generations. This frequency was increased by ethyl methanesulfonate (EMS) treatment to $3x10^{-5}$ substitutions per site per generation. EMS is a mutagenic, teratogenic, and carcinogenic organic compound with formula $C_3H_8SO_3$ which produces random mutations, mostly G:C to A:T transitions induced by guanine alkylation, in genetic material by nucleotide substitution. EMS typically produces only point mutations. Genetic alterations due to physical and chemical mutagens can be classified as genome, chromosome, and gene mutations [31, 35, 39–42].

Genome mutations not only affect genome size (ploidy) but also genome rearrangement in plants. Many plant species as bread wheat: 6X; durum wheat: 4X, cotton: 4X, potato: 4X have polyploidy in nature. Polyploidy leads various advantages as enhanced nucleus size, enlargement on cell and organism basis, yield, increase in gene variations. Polyploidy can be induced as genome duplication (autoploidy) and increase in genome size (alloploidy) through use of mutagens [34, 43].

Chromosome mutations occur during meiotic cell division in very low frequencies. In euploidy state of plants, one set of chromosomes are present, while radiation exposure may result whole or partial chromosome deletions, insertions or translocations and cause aneuploidy. Besides, chromosome inversions, which are characterized as a chromosome rearrangement in which a segment of a chromosome is reversed 180 degrees end-to-end, cause very high gene recombination. In chromosome translocations, break off chromosome parts may attach to the same chromosome (intrachromosomal) or different chromosome (inter-chromosomal). Both, inter- and intra-chromosomal translocations lead to devastating effects on gene expression.

Gene mutations can be either as gene copy number alterations or as point-mutations, insertions, deletions on nucleotides of gene sequence. Plants may increase gene copy numbers to enhance protein expression during metabolic functions. Mutagens can affect gene expression profiles through either by increasing or decreasing gene copy numbers. Point-mutations occur particularly in chemical mutagen applications. Single or set of nucleoid alterations cause silent mutations if they do not occur in genic regions. Alternatively, they can also cause nonfunctional gene products or nonsense mutations. Nucleotide insertions or deletions can alter codon structure and cause shift in open reading frames. These alterations can also occur on promotor regions, coding sequences or intron regions of genes, therefore, significantly effect protein expression [34, 35].

Single nucleotide changes as deletions generally cause functional gene mutations by the leading formation of novel alleles. Hence, they are particularly important for plant breeding studies for inducing genetic variations. There are numerous examples of plant height, abiotic stress tolerance, pesticide and herbicide resistance improvement cases in rice, wheat, barley, soybean plants, and more [44–47]. Nucleotide mutations can also occur in non-genic regions and cause silent mutations which have

no apparent effect on gene expression. Silent mutations generally occur following the alkylating chemical applications and do not affect translation [37]. Deletions among intergenic regions remain silent as long as they do not affect regulating sequences. Still, the possibility of open reading frame shift is present and may lead to nonfunctioning peptide formation [35].

In mutation breeding studies, whole plant, meristem tips, pollens, in vitro explants, embryos, microspores, callus cultures can be selected as initial materials. However, seeds are mostly preferred materials by plant breeders due to the advantages as metabolic inactivity, easy transport, ease of application, low space requirement, ease of storage comparing to others.

2.3.1 Mutagens

Choice of appropriate mutagen is one of the deciding factors on succession of the mutation breeding program. Physical, chemical, or biological agents are viable alternatives. Among physical mutagens ionizing radiation sources, particle (electrons, protons, neutrons, alpha and beta particles) or electromagnetic (X-rays, gamma rays), are widely used. Ionizing radiation interacts with genetic material and cause mutations on DNA sequences. Magnitude of mutagenic effect is proportional to the radiation dose. It is crucial to determine and optimize the effective radiation dose based on experimental plant variety, plant part, and radiation source. 80% of mutation breeding studies prefer physical mutagens and of 60% of this use gamma radiation [35].

Chemical mutagens offer much larger alternative choices. However, the most widespread use of chemical mutagens is among alkylating agents. Ethyl methane sulphonate (EMS), diethyl sulphate (DES), ethylene imine (EI), N-ethyl-N-nitroso urea (ENU), ethyl nitrite urethane (ENU), N-methyl-N-nitrosourea (MNU) are the most generally preferred chemicals. O^6-alkylguanine, N^3-alkyladenine, N^3-alkylcitosine leads to alternative allele formation. Besides methylating agents, nitric acid, nucleic acid analogs, some antibiotics (streptozotocin, mitomycin C, azaserine) are other important chemical mutagens. 60% of registered chemically induced mutant plants are developed by use of EMS, MNU and EMU. One-third of these mutants are obtained by EMU which has ease of supply among others.

Among physical mutagens, gamma radiation has the most frequent use. In nature, there are various gamma-emitting isotopes such as potassium-40 (^{40}K), however, in plant breeding applications cobalt-60 (^{60}Co) and cesium-137 (^{137}Cs) are the common choices.

In the last 20 years, there are 599 different developed mutant plants belonging to 78 different plant species registered to International Atomic Energy Agency (IAEA) Mutant Variety Database [48]. Soybean is in the third place among these plants with 46 registered mutants (8%) after 247 rice (42%) and 55 wheat (9%) mutants. In the category referred as others, chickpea, carnation, tomato, mung bean, Hibiscus, rapeseed, sesame, orchid, pepper, cowpea, glory bush and sunflower have the most mutants (**Figure 1**).

Among the soybean mutants, there are 15 different improved traits. They can be listed as; high yield, high protein content, resistance to soybean mosaic virus (SMV), early maturity, resistance to leaf rust, resistance to purple seed stain, resistance to cyst nematode (SCN), resistance to lodging, drought tolerance, super nodulation, absence of lipoxygenase, temperature tolerance, low allergenicity and higher nitrogen fixation (**Figure 2**). Thirty-six of these traits were improved by the use of gamma rays
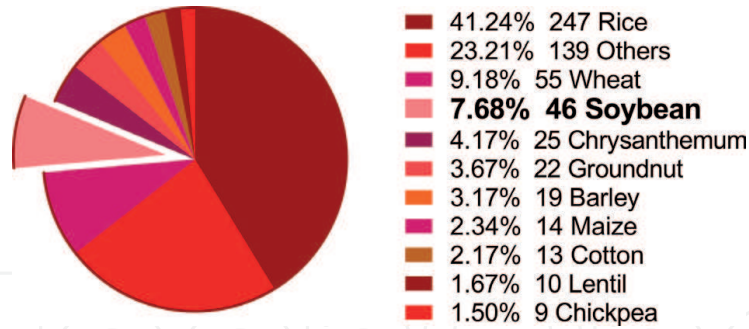
Figure 1.
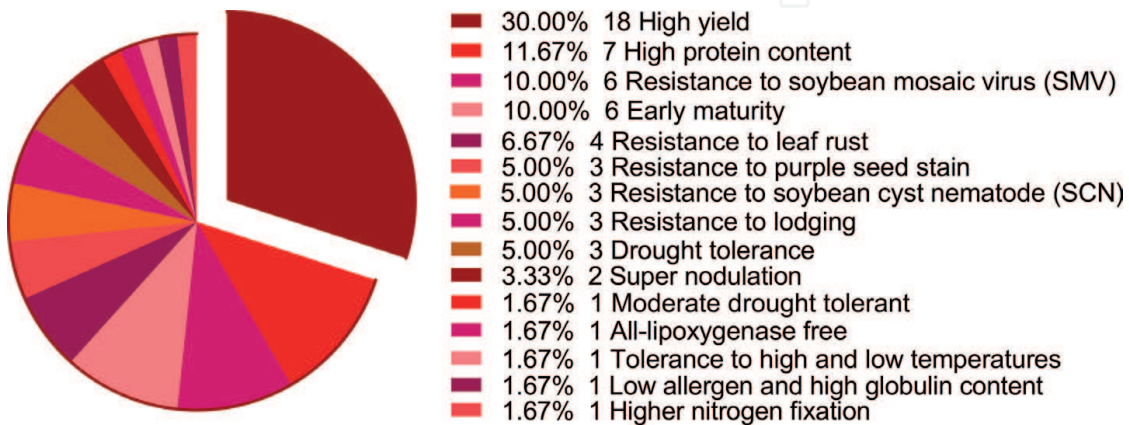*Mutation variety database of IAEA registered mutant plants in last 20 years [48].*

Figure 2.
Radiation-induced trait improvements achieved and registered to MVD in last 20 years.

as physical mutagens, while 7 of them were developed by chemical mutagens. In this period, China is the leading country with 9 registered soybean mutants while Japan (9), Viet Nam (5), Bulgaria (3), India (3), Indonesia (3), Republic of Moldova (3) Republic of Korea (1) and Thailand (1) are the followers.

2.3.2 Present applications of mutation breeding in soybean

In the last decades of mutation breeding, radiosensitivity of different plant species and tissues were investigated and dose limits were determined for various plants. In present days, molecular marker-based techniques were widely applied to estimate genetic diversity and population structure. Among these techniques restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), and inter-simple sequence repeats (ISSRs) are viable options depending on the advantages and limitations of each technique. SNPs, which are spread across in both non-coding and coding regions of the genome, are also preferred in many mutation studies [49]. Present applications of the marker-based techniques include even transposable elements (TEs). The target region amplification polymorphism (TRAP) is a novel, polymerase chain reaction (PCR)-based marker system which exploits the available EST database sequence data to generate polymorphic markers targeting candidate genes. This method utilizes an 18-mer primer derived from the EST sequence and pairs it with an arbitrary primer that targets the intron and/or exon region. TRAP method is useful for germplasm genotyping and producing markers associated with desirable

agronomic traits in mutation breeding. Hung et al. [50] employed this simple rapid method by using the consensus terminal inverted repeat sequences of PONG, miniature inverted-repeat transposable element (MITE)-Tourist (M-t) and MITE-Stowaway (M-s) as target region amplification polymorphism (TE-TRAP) markers to investigate the mobility of TEs in a gamma-irradiated soybean mutant pool. They concluded that MITEs were significant enough to confirm their practical utility as molecular markers for investigating mutant populations which were induced by random variations caused through physical mutagenesis (X-ray or gamma-ray). Also, the TE-TRAP marker system was suggested as it provides a simple, rapid, and cost-effective alternative for investigating genetic diversity and identifying mutant lines in irradiated soybean mutant breeding. Kim et al. [51] conducted a genetic diversity and association analysis of soybean mutants to assess elite mutant lines which were induced by 250 Gy of gamma rays using a ^{60}Co gamma-irradiator. They have chosen 208 soybean mutants by phenotypic traits to mutant diversity pool (MDP) and investigated the genetic diversity and inter-relationships of these MDP lines using TRAP markers. MDP has been suggested to have great potential for soybean genetic resources. TRAP markers were found useful for the selection of soybean mutants in mutation breeding applications [51].

Besides the genetic diversity and population structure analysis, genetic characterization of improved mutants and the determination of the source of the gained trait in sequence basis studies have taken over the course of mutation breeding in present days. Before the genomic era which was ignited through the breakthrough discovery of DNA sequencing by Sanger et al. [52], the heteroduplex mismatch cleavage assay which is based on mismatch-specific endonuclease Cel I, was the standard method to detect point mutations. As a simple, rapid, and cheaper mutant discovery method, high resolution melting (HRM) analysis was applied to many agronomical crops. Following the Sanger sequencing, the final step of mutation screening was changed to Sanger to evaluate the changes in the genome and effects of mutation on amino acid substitutions. Today, next generation sequencing (NGS) technologies are the gold standard in the mutation detection field with various options as Roche 454 pyrosequencing, sequencing-by-synthesis, SOLiD sequencing and the HiSeq 2000, which is the gold standard of high-throughput sequencing. Tsuda et al. [53] reported the construction of a high-density mutant library in soybean and the development of a mutant retrieval method referred as amplicon sequencing which is an alternative, cheaper method for sequencing the PCR amplicons in targeted regions. The library of DNA and seeds of EMS-induced plants revealed large morphological and physiological variations. They retrieved the mutants through HRM and indexed amplicon sequencing analysis and confirmed by Sanger sequencing in the final step. They concluded that indexed amplicon sequencing allows researchers to scan a longer sequence range and skip screening steps and also, to know the sequence information of mutation due to the utilization of systematic DNA pooling and the index of NGS reads, which simplifies the discovery of mutants with amino acid substitutions comparing to the HRM screening [53].

MutMap method which utilizes the sequencing technique for mapping the mutated genes responsible for the desired trait was introduced for mutation breeding studies. The first application of the method has been developed by Abe et al. [54] to identify the mutated gene responsible for the change in leaf color from dark green to light green in rice [54]. Thereafter, it has been commonly used for mapping the monogenic recessive genes. In this method, a cultivar with a known reference sequence can be mutagenized by either chemical or physical mutagens. After the

selfing and homozygosity experiment for the desired trait between M_3 to M_6 generations, mutants are crossed with their parental or wild type varieties. F_2 population is obtained by selfing of F_1. If the desired trait is inherited through a single recessive gene, the segregation ratio should be of 3:1 in wild and mutant phenotype in F_2 population. In MutMap method, DNA of homozygous mutant plants are extracted and subjected to whole genome sequencing. The mutant genomes are compared to the publicly accessible reference sequences to determine single nucleotide polymorphic (SNPs) variations. The linkage between mutants and wild type plants can be evaluated according to SNP ratios in which the ratio infer that the SNP variation is not linked to the mutation if ranged between 0.1 and 0.5, while it can be linked to the mutation when ranges are between 0.51 to 1 [54, 55]. Kato et al. [56] introduced Lumi-Map, which is a high-throughput platform for identifying causative SNPs for studying pathogen-associated molecular patterns (PAMP) triggered immunity (PTI) signaling components, in combination with MutMap. In Lumi-Map method, they generated nine transgenic *Arabidopsis* reporter lines expressing the LUC gene fused to multiple promoter sequences of defense-related genes, that generates luminescence upon activation of FLAGELLIN-SENSING 2 (FLS2) by flg22, a PAMP derived from bacterial flagellin treatment. Mutagenesis of the line as achieved through EMS treatment and the mutants with altered luminescence patterns were screened by a high-throughput real-time bioluminescence monitoring system. They subjected MutMap method on selected mutants to identify the causative SNP responsible for the luminescence pattern alterations. WRKY29-promoter reporter line was selected to identify mutants in the signaling pathway downstream of FLS2. Twenty-two mutants with altered WRKY29 expression upon flg22 treatment among 24,000 EMS-induced mutants of the reporter line were isolated. In this mutagenesis study, Lumi-Map method combined with MutMap revealed three genes not previously associated with PTI and suggested as a potential alternative to identify novel PAMPs and their receptors as well as signaling components downstream of the receptors [56]. Takagi et al. [57] exploited the rapid and versatile properties of MutMap for more than 20,000 ha of rice paddy field which was inundated with seawater, resulting in salt contamination of the land in Japan following the 2011 earthquake and tsunami that affected Japan. They needed an improved rice variety at short notice as local rice landraces were not tolerant of high salt concentrations caused by seawater. They obtained 6000 EMS-induced mutant lines of a local elite cultivar, 'Hitomebore'. MutMap method was used to rapidly identify a loss-of-function mutation responsible for the salt tolerance of hst1 rice. The detected salt-tolerant hst1 mutant was used to breed a salt-tolerant Kaijin variety which differs from Hitomebore by only 201 SNPs. Conducted field trials presented that improved variety had the equal growth and yield performance as the parental line under normal growth conditions. The whole process was completed only in 2 years which proves the efficiency of MutMap in mutation breeding studies [57]. Fekih et al. [58] improved the method even further and introduced the MutMap+ which is a modified version of MutMap developed for the cases in which obtaining F_2 mapping population is impossible due to the lethal mutations or sterility. MutMap+ has advantages over MutMap as it is less complex, time-consuming, and costly especially in large mapping population. Also, hybridization step of MutMap can be relatively compelling especially in small flower plant species and in crops that are recalcitrant to artificial crosses, therefore, MutMap+, which notably does not necessitate artificial crossing between mutants and the wild-type parental line, is advantageous. In MutMap+ method, again, a cultivar with known reference sequence can be mutagenized by either chemical or physical mutagens. M_1 plants are selfed to develop

M_2 generation. However, in MutMap+ mutants are not crossed with their parental or wild type varieties. The heterozygous M_2 mutant plants are selfed to develop M_3 generation in which the segregation ratio of 3:1 for wild and mutant phenotype is expected. DNAs of tagged mutants and parental varieties are extracted, and pooled. Following the whole genome sequencing, data is compared to the reference genome and SNP profiles are determined. They identified causal nucleotide changes of rice mutants of NAP6 gene that is responsible for change in leaf color and consequent lethality after germination. This versatile extension of MutMap method, also allow determination of recessive lethal alleles [58].

In soybean, Liu et al. [59] investigated two types of resistant sources which are widely used against soybean cyst nematode (SCN, *Heterodera glycines* Ichinohe). Peking-type soybean requires both rhg1-a and Rhg4 alleles, while PI 88788-type soybean requires only the rhg1-b allele for resistance. Instead of MutMap, they preferred the region-specific extraction sequencing (RSE-Seq) method which is developed to enrich a targeted chromosomal segment for genome sequencing to identify SCN resistance genes within the identified 300 kb chromosomal segment carrying the rhg1 locus, due to the requirement of MutMap to an additional procedure of backcross of phenotypic mutants with the wild-type. They suggested GmSNAP18 gene as a candidate for the resistance of two various resistant types of soybeans for SCN [59]. RSE is a cost-effective, long-range DNA target capture methodology that relies on the specific hybridization of short (20–25 base) oligonucleotide primers to selected sequence motifs within the DNA target region This target enrichment method can produce sequencing templates more than 20 kbp in length. These capture primers are then enzymatically extended on the 3′-end, incorporating biotinylated nucleotides into DNA. Streptavidin-coated beads are subsequently used to pull down the original, long DNA template molecules through synthesized, biotinylated DNA that is bound to them [60]. QTL-seq is another method adapted from MutMap to identify quantitative trait loci. In presence of pooled two segregating progeny populations with opposite traits as resistant and susceptible and single whole-genome resequencing of either of the parental cultivars, it utilizes pooled sequences. Also, modified QTL-seq using high-resolution mapping has been developed to cover the weakness of original QTL-seq which do not assume a highly heterozygous genome [61]. Direct whole genome re-sequencing (WGRS) is also utilized effectively to identify candidate genes involved in resistance to SCN in soybean due to the requirement of time-consuming backcrosses in MutMap and QTL-seq methods. Two EMS-induced soybean mutants and six relevant whole genomes were re-sequenced to determine genomic variants as SNPs and InDels. Comparison by this method eliminated many genomic variants from the mutant lines that overlapped non-phenotypic but mutant progeny plants. Therefore, the method was suggested as simple but effective to the identify other trait genes in soybean, even in other organisms [62]. Likewise, comparative genomic analyses of two segregating soybean mutants which were selected among 500 EMS-induced candidates revealed seven genes potentially involved in resistance to *Fusarium equiseti* through WGRS. These genes were suggested to facilitate the breeding of resistant germplasm resources and the identification of resistance to *Fusarium spp*. in soybean [63].

3. Future Prospect and conclusion

Soybean genetic variation improvement is important for the development of superior cultivars. One of the greatest challenges in mutation breeding is random

İntended Development	Target Gene/Site/ Protein	Soybean Line	Transformation Method	Other Model Organizm	gRNAs	Referance
Soybean cyst nematode (SCN) (*Heterodera glycines*) resistance	Glyma.15G191200/γ-SNAP Protein	LD10–30110 Resistance / LD10–30080 and LD10–30092 Susceptible	*Agrobacterium rhizogenes* strain ARqua 1	*N. benthamiana*	TCCGCTGCTGCTCTCGCAA and GTATTCTGTTGCAGCTAAT	[64]
Soybean cytoplasmic malesterile (CMS)	The Aborted Microspores (AMS) Gene	Williams 82	*Agrobacterium tumefaciens* strain EHA105	—	NA	[65]
Heat Stress Tolerance	Glyma.16G178800.1/ Hsp90A2	TianLong No. 1	*A. tumefaciens* strain EHA101	Yeast strain NMY51	NA	[66]
Salt Stress Tolerance	Glyma.06 g21020.1/ NAC06	Williams 82	*A. rhizogenes* strain K599	Yeast strain AH109	NA	[67]
Fatty Acid Content	Glyma.10G278000/ FAD2–1A and Glyma.20G111000/ FAD2–1B	Williams 82 and Maverick	*A. rhizogenesis* strain K599	—	CCAAACACAAAGCCACCATTCAC and GATGAAGGAACATCCGAGAA	[68]
Flowering time and plant height	Glyma.16G091300/ APETALA1 (AP1)	Williams 82 and HX3	*A. tumefaciens* strain EHA101	*Nicotiana benthamiana*	NA	[69]
Flowering Time	Glyma.16 g26660/FT2a	Jack	A. tumefaciens strain EHA105	—	GTAGGGATCCTCTCGTTGTTGGG	[70]
Lipoxygenase-Free	Glyma.13 g347600, Glyma.13 g347500, and Glyma.15 g026300/ LOX1, LOX2, and LOX3	Huachun 6/ Lipoxygenase Free Cultivar Wuxing 4	*A. tumefaciens* strains GV3101	—	GGAAAGGATACGTTCTTG GAAGG(sgRNA(GmLox1/2)/ CCTTTCCTTATCCTCGTAGGGGG(sgRNA-GmLox3)	[71]

İntended Development	Target Gene/Site/ Protein	Soybean Line	Transformation Method	Other Model Organizm	gRNAs	Referance
Decreased Allergenic Genes	Glyma.U020300.1/ Bd 28 K and Glyma.08G116300.1/ Bd 30 K	Enrei and Kariyutaka	*A. tumefaciens* EHA105	—	CCACTCAGCGAACCGGATATTGG and ACCCAAGTAAAGTACCAAGGGGG	[72]
Increases İsoflavone Content	Glyma.11G253000/ Phytoene Desaturase (PDS) and Glyma.10G278000/FAD2	Jack	*A. rhizogenes* strain K599	—	GAAGCAAGAGACGTTCTAGGTGG and AGTTGGCCAACAGTGAATGGTGG	[73]
Soybean Mosaic Virus (SMV)	Glyma.04G196100/ Asetolaktat sentaz (ALS)	Williams82	*A. tumefaciens* strain EHA105	—	CGTCGGCGAGGCCGCTCACGAGG	[73]
Reduced Saturated Fatty Acids	Glyma.05G012300/ FATB1a and Glyma.17G012400/ FATB1b	Williams 82	*Agrobacterium strain tumefaciens* LBA4404	*Arabidopsis thaliana*	GTTAAAAGTGCTGGGCTTCTTGG and GTTAAAAGTGCTGGGCTTCT	[74]
Early Flowering	Glyma.06G207800/E1 Protein	Jack	*A. tumefaciens* strain EHA105	—	CCCTTCAGATGAAAGGGAGCAGT and CCACCATATGCGAAGCCTCTAAC	[75]
Seed Storage	Glyma.20 g148400/ Conglycinins (7S) and Glyma.03 g163500/ Glycinins (11S)	Harosoy 63	*A. rhizogenes* strain K599	—	CCTTCTGAT GAGGTG GGC GT and GATAAC CGTATAGAGTCAGA	[76]
Architecture in Soybean	Glyma.02G177500/ Squamosa Promoter Binding-like Proteın 9 (Spl9a)	Williams 82	*A. tumefaciens* strain EHA105	—	TCCCTTGATGGCTTGAAGTTTGG	[76]
Drought Stress	Glyma.16G151500/NAC8	Tianlong No.1	*A. tumefaciens* strain EHA101	*Nicotiana benthamiana*	CCATCTTATCTGAGAACCACTCC	[77]
Herbicide Resistant	DD20 and DD43	93B86	Particle Bombardment	—	GGAACTGACACACGACATGATGG	[78]

Table 1.
Targeted mutagenesis application examples for soybean.

(uncontrolled) nature of induced mutagenesis. Large population requirement for desired mutant selection brings intensive labor. The emergence of clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) technology has brought wider insight to the field through allowing targeted mutagenesis. It has been widely used in numerous plants as rice, wheat, maize, oilseed rape, barley, cotton, tomato and soybean as well. However, utilization of CRISPR/Cas9 system in soybean is still limited due to the transformation challenges in soybean. As summarized in **Table 1**, most of the targets which were successfully applied to soybean were single gene edits. Paleopolyploid genome of soybean in which approximately 75% of the genes have multiple copies, requires multiple genes or paralogous genes to regulate many important traits. Therefore, these traits may only be targeted by editing which requires the engineering of homologous sequences using more than one sgRNA for recognition. Introducing multiple constructs simultaneously to soybean is relatively limiting in terms of genome editing associated soybean breeding approaches. Recently, Zhang et al. [73] successfully optimized one sgRNA CRISPR/Cas9 system in soybean for the target-specific mutations at multiple loci of GmFAD2 and GmALS. They evaluated the efficiency, type, specificity, and patterns of multiple targeted mutations by selecting three different genes with known functions in soybean and suggested that CRISPR/Cas9 could specifically and efficiently induce targeted mutations at one locus or multiple loci in the T_0 generation. Moreover, they demonstrated the necessity of simultaneous modification of different homoeologous gene copies in polyploid soybean for successful CRISPR-Cas9-mediated breeding [73]. Therefore, induced mutagenesis is still a major method to produce new alleles and new desired traits within the crop genomes. Physical and chemical mutagen protocols are still improving and mutation breeding proves its value to be fast, flexible, and viable in crop sciences.

The second most limiting prospect of induced mutagenesis was the requirement of at least three generation before any stable selection of desired traits in mutants which leads to 7−9 years of average mutation breeding study, previously. However, as described in previous sections NGS based approaches as MutMap accelerated the selection periods significantly. Novel non-destructive measurement methods allow automated imaging and optical measurements of the same plants for desired periods. These approaches provide high measurement densities and fill the gap between genotype and phenotype in mutation breeding studies which is still another limitation in this field. Repeated imaging of particular genotypes under different environmental conditions leads to the generation of development models for biologically relevant parameters. In the present omics era, future procedures may shorten the selection procedures even further [79].

In conclusion, mutation breeding passed important cross-roads successfully during recent advances in plant biotechnology, transformation and targeted mutagenesis by its particular great advantages. Mutagenesis will retain its place in crop science in next decades especially for the plants as soybean for which cross breeding is limited or not applicable.

Author details

Alp Ayan*, Sinan Meriç, Tamer Gümüş and Çimen Atak
Faculty of Science and Letters, Department of Molecular Biology and Genetic,
Istanbul Kultur University, Istanbul, Turkey

*Address all correspondence to: a.ayan@iku.edu.tr

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