Mutagenesis and TILLING to Dissect Gene Function in Plants

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Abstract: Mutagenesis can be random or targeted and occur by nature or artificially by humans. However, the bulk of mutagenesis employed in plants are random and caused by physical agents such as x-ray and gamma-ray or chemicals such as ethyl-methane sulfonate (EMS). Researchers are interested in first identifying these mutations and/or polymorphisms in the genome followed by investigating their effects in the plant function as well as their application in crop improvement. The high-throughput technique called TILLING (Targeting Induced Local Lesion IN Genomes) has been already established and become popular for identifying candidate mutant individuals harboring mutations in the gene of interest. TILLING is a non-transgenic and reverse genetics method of identifying a single nucleotide changes. The procedure of TILLING comprises traditional mutagenesis using optimum type and concentration of mutagen, development of a non-chimeric population, DNA extraction and pooling, mutation detection as well as validation of results. In general, TILLING has proved to be robust in identifying useful mutant lines in diverse economically important crops of the world. The main goal of the current mini-review is to show the significance role played by mutagenesis and TILLING in the discovery of DNA lesions which are to be used in the improvement of crops for the trait of interest.

Keywords: Ethyl methane sulfonate, High-throughput technique, Mutagenesis, Mutation induction, Mutation detection, Mutation breeding, Targeted mutagenesis, TILLING.

1. INTRODUCTION

Development of improved crop varieties is time bound and depends on availability of diverse techniques. The improvement of the current crop varieties took long time and diverse techniques. Artificial selection is an ancient method that are still in use to improve crop plants. The type of selection technique to be implemented depends mainly on the mating system of the crop. In addition to selection, hybridization is a widely applied technique since it utilizes the variability created during crossing for the benefit of crop improvement. Hybridization (also known as introgression) between two parents is either an intra-specific (crossing within the same species) or inter-specific (crossing between different species). Plant tissue culture particularly the in vitro regeneration of plants from cells, tissues and organs has been successfully implemented in diverse types of crops including cereals [1, 2], legumes [3], vegetables [4, 5], oil plants [6], fruits [7], trees [8], and forestry [9]. Diverse marker-assisted selection (MAS) techniques have been utilized to effectively assemble favorable alleles in phenotypic selection [10]. The most common genetic markers are SSRs (Simple Sequence Repeats, or microsatellites) and SNPs (Single Nucleotide Polymorphisms). SSRs refer to a repeat of 2-6 nucleotides in the DNA sequences which are highly polymorphic and abundant in the genomes of organisms. SNP is a type of polymorphism in which a single nucleotide difference is present among genotypes.

Transgenic technology is also considered as an alternative approach of improving crop productivity. The global area under transgenic crops has been rapidly increasing from just 1.7 million ha in 1996 to 181 million hectares in 2014 [11]. This over 100-fold increase in less than 20 years makes this technology among the most adopted technologies in agriculture. Despite its rapid adoption and extensive cultivation in USA, Brazil, Argentina and Canada, there is big concern and protest against transgenics in Europe and Africa.

Mutation breeding using either physical or chemical agents has been extensively applied by breeders for the last 70-80 years. Mutations created by diverse mutagens were the base to develop for the release of over 3000 crop varieties globally [12]. Most mutation breeding programs were aimed at altering traits such as plant height and disease resistance in well-adapted plant varieties of rice, barley, and wheat [12].

High-throughput techniques which investigate natural and induced mutations can effectively identify the mutation or polymorphism in the altered gene. This enables plant breeders to select the trait of interest and integrate into the
breeding program which will be finally released to the farming community. A reverse-genetics technique called TILLING (Targeting Induced Local lesions IN Genomes) accompanies a traditional mutagenesis and a high-throughput screening in order to identify mutant genotypes harboring mutations in the gene of interest. In addition to its high efficiency in mutation detection, TILLING has received high acceptance by the public since the products developed using this technique are exempted from the biosafety regulations imposed on transgenics. In this review, the significance of mutagenesis and TILLING as well as their application to crop improvement are discussed.

2. MUTAGENESIS: THE SOURCE OF GENETIC VARIABILITY AND CROP IMPROVEMENT

Mutagenesis refers to the stable and heritable alteration of the genetic material of the organism. Although mutagenesis normally refers to the creation of mutation, three categories are identified especially considering the utilization of mutations in crop improvement. These are mutation induction, mutation detection and mutation breeding [13].

2.1. Mutation Induction

Mutation induction which refers to the creation of genetic diversity can be investigated based on at least four aspects: i) source of mutation (natural or induced), ii) type of mutagen (physical or chemical), iii) patterns of DNA cleavage intensity of mutation (point mutation, INDELs or rearrangements) and spectrum of mutation (nonsense, missense, silent or splice junction), and iv) precision of the mutation (random or targeted). (Table 1) shows diverse types of mutations, mutagens and detection methods applied in plants.

2.1.1. Sources of Mutation

Mutations are either caused by natural or man-made agents. Natural mutation mainly occurs due to error in DNA replication and physical agents while induced mutation is caused by physical or chemical agents. The advantages and disadvantages as well as key features of various sources of mutation were reviewed [14].

2.1.2. Type of Mutagen

Mutation breeding relies on the implementation of either physical or chemical agents in order to create variability in the population of interest. Commonly used physical mutagens are ionizing radiations which include gamma-ray, x-ray, and fast neutron, and a non-ionizing radiation (e.g. UV). These physical mutagens cause diverse types of damages to the exposed organism. Widely used chemical mutagens are ethyl methane sulfonate (EMS), N-methyl-N-nitrosourea (MNU) and sodium azide (Az) [15, 16]. While chemical mutagens such as EMS mainly create a point muta-

| Source of Mutation                  | Agent/cause                         | Effect of Mutation | Mutation Detection Technique | Remarks             | References |
|-------------------------------------|-------------------------------------|--------------------|------------------------------|---------------------|------------|
| Natural                             |                                     |                    |                              |                     |            |
| Diverse                             |                                     | Point mutation     | EcoTILLING, GBS, RAD         |                     | [37-39]    |
| Diverse                             |                                     | INDELs             | AFLP, SSR                    |                     | [35, 85]   |
| Induced                             |                                     |                    |                              |                     |            |
| Random mutation                     | Chemicals (EMS, NaN₃, MNU, ENU)     | Point mutation     | Mutmap, Map-based            | Forward-genetics    | [40]       |
|                                     | Chemical or physical                | Point mutation     | TILLING                      | Reverse genetics    | [19]       |
| Ionizing radiation (x-ray, gamma-ray)| DNA strand breakage                |                    |                              |                     |            |
| Fast neutron                        | Large DNA damage                    | Deleteagene        |                              |                     | [17]       |
| Non-ionizing radiation (UV)         | Pyrimidine dimer                    |                    |                              |                     |            |
| Insertional mutagenesis (T-DNA)     | TAIL-PCR                            |                    |                              |                     | [41]       |
| Targeted mutation                   | Meganuclease                        | DSB & repair       |                              |                     | [21]       |
| (Genome editing)                    | ZFN                                 | DSB & repair       |                              |                     | [22, 23]   |
|                                     | TALEN                               | DSB & repair       |                              |                     | [24, 25]   |
|                                     | CRISPR/Cas9                         | DSB & repair       |                              |                     | [26-28]    |
tion in which a single nucleotide is altered, physical mutagens such as fast neutron remove large pieces of DNA which could be detected using a Deleagene technique [17]. The advantage of the chemical mutagenesis is that it creates an allelic series of mutations.

2.1.3. Patterns of DNA Cleavage Intensity and Spectrum of Mutation

The effect of mutation on a plant genome range from a point mutation with limited phenotypic change to large deletions or insertions which result in deformity or lethality of the whole plant. Gross alterations in some parts of the genome which include deletions, duplications, inversions and translocations are commonly referred to re-arrangement [18].

The following spectrum of point mutations were reported: i) **nonsense mutations**: a single base pair change which converts an amino acid codon into a stop codon, ii) **missense mutations**: a single base pair change that alters the amino acid encoded by a particular codon, iii) **silent mutations**: a single base pair change which does not alter the amino acid encoded by a particular codon, and iv) **splicing junction mutations**: a single base pair change that alters the canonical GT/AG splice sites and results in a truncated protein [19]. Studies made on mutation spectrum and nucleotide substitutions of diverse crops were documented [20].

2.1.4. Precision of the Mutation

- **Random mutagenesis**: the bulk of mutations occurring due to both natural and induced mutations are random. Except for recently discovered Genome Editing tools, the majority of induced mutation integrate in plant genome at random position. These random mutagenesis are mostly caused by chemicals, physical agents and insertional mutagenesis such as T-DNA and transposons.

- **Targeted mutagenesis**: also known as ‘Genome Editing’ is based on nucleases that create specific double-stranded break at desired locations in the genome; hence can directly modify a gene of interest within a genome. So far, four types of nucleases with diverse efficiency are known. These are meganuclease or homing endonucleases [21], Zinc Finger Nucleases (ZFNs) [22, 23], Transcription Activator-Like Effector Nucleases (TALENs) [24, 25], and the Clustered regularly interspaced short palindromic repeats / CRISPR-associated9 (CRISPR/Cas9) [26-28]. While the last three methods are efficient and specific, CRISPR/Cas9 has additional benefits as it targets multiple sites simultaneously unlike ZFNs and TALENs [14]. Hence, CRISPR/Cas9 has been considered as a method of choice. Although the products of genome-editing are free of transgene since they lost foreign DNA due to random assortment and chromosome segregation [29], it is difficult to speculate whether the bio-safety law imposed on GMOs will also be applied to the products of Genome Editing. A list of genes modified in model and crop plants using the three genome-editing methods are available [30, 31]. At present, there are at least two web-based services for the plant community interested to use the latest genome-editing system. These are, i) CRISPR-Plant, hosted by the Arizona Genomics Institute, which provides service for eight plants including rice, maize, sorghum, soybean and tomato [32], and ii) CRISPR-P, hosted by Huazhong Agricultural University in China, which provides service for 34 plant species including Brassica, rice, potato and tomato [33, 34].

2.2. Mutation Detection

2.2.1. Detection of Natural Mutations

Various tools have been implemented to identify polymorphism in plants. AFLP (Amplified Fragment Length Polymorphisms) which detects DNA polymorphism using restriction enzyme digestion of DNA and selective amplification of DNA fragments, has been widely implemented in diverse crops [35]. SSRs (also known as microsatellites) are 2-6 nucleotide repeats which are abundantly distributed throughout the genome and are highly polymorphic [36]. GBS (Genotyping-by-sequencing) and RAD (Restriction-site Associated DNA) apply the high-throughput sequencing to study SNP diversity among diverse crop genotypes [37, 38]. EcoTILLING which implements similar protocol to the TILLING (described in detail below) is the high-throughput technique which identify polymorphism for the gene of interest among natural populations [39].

2.2.2. Detection of Induced Mutations

In the forward genetics system, point mutations can be revealed by the lengthy map-based system or by the recently discovered MutMap technique [40]. Mutmap is proved to identify unique genomic position harboring mutations in semi-dwarism in rice [40]. In this case, a mutant line with desirable trait was first crossed to the original line followed by self-pollination in order to obtain F2 progenies for SNPs discovery. Regarding insertional mutagenesis, the gene responsible for the altered phenotype can be isolated using the Thermal Asymmetric Interlaced PCR (TAIL-PCR) technique which rely on the nested PCR technique which use degenerate primers [41].

2.3. Mutation Breeding

During the last seventy years, mutation breeding contributed significantly to the improvement of many economically important crops. Crops descended from this technique were superior to the original cultivars in productivity and/or tolerance to biotic and abiotic stresses. The lists of officially released and/or commercially available crop varieties originated from induced mutation are available by the Mutant Variety Database (MVD) of the Joint IAEA/FAO Program [12]. According to the database, more than 3200 mutant varieties of which 50% are cereals have been officially released in more than 210 plant species from more than 70 countries. By searching in the data base, crop varieties with improved traits can easily be identified. Hence, researchers can save their time and resources provided free exchange of these elite materials is guaranteed.

3. TILLING: A HIGH-THROUGHPUT MUTATION DETECTION TECHNIQUE

Some benefits of TILLING are: i) it produces a spectrum of allelic mutations that are useful for genetic analysis; ii) mutations that are difficult to know by the forward genetics could be revealed since TILLING can focus at a particular
gene of interest; iii) it applies to any organism with different genome size and ploidy level; iv) it produces stable mutation; and v) since no exogenous DNA is introduced into the plant, the product is considered as a non-transgenic and is exempted from regulatory restrictions or procedures imposed on transgenic products [42, 43].

So far, TILLING has successfully been implemented in diverse types of crops which include cereals (maize [44], wheat [45-47], rice [48-51], barley [52-54], sorghum [55] and tef [43]); legumes (pea [56]); vegetables (potato [57] and tomato [58, 59]); and oilseeds (soybean [60] and canola [61]). The list of TILLING populations and platforms established for diverse model and crop plants is available [20, 62].

The technique of TILLING comprises the following main steps: i) mutagenesis, ii) development of a non-chimeric population, iii) preparation of a germplasm stock, iv) DNA extraction and sample pooling, v) population screening to detect mutation in the desired gene, and vi) identification of mutant line and sequencing the target gene [63].

3.1. Major Components of TILLING Procedure

3.1.1. Identifying Experimental Material

While the majority of TILLING projects use seeds for mutagenesis, few others choose other part of the plant. For example, the Maize TILLING was performed on the M 1 mutant line and sequencing the target gene [63]. To detect mutation in the desired gene, and vi) identification of mutant line and sequencing the target gene [63].

3.1.2. Determining the Type and Concentration of the Mutagen

Broadly, mutagens are grouped into chemical and physical agents. Among physical mutagens gamma-ray, x-ray, and fast neutrons are widely used. Commonly used chemical mutagens include ethyl methane sulfonate (EMS), sodium azide (NaN3) and N-methyl-N-nitrosourea (MNU). In general, mutagens are known to create diverse types of mutations in the genome which range from point mutations with no phenotypic change to DNA strand breaks which lead to the genetic instability of the organism. Since TILLING can be directly implemented on improved or elite cultivars, it avoids the need for introgression of a mutant allele to high-yielding varieties. Hence, the introduction of undesirable traits is reduced if not eliminated [47, 66].

3.1.3. Creating Mutagenized Population

Once the type and optimum concentration of the mutagen is identified, large-scale mutagenesis is implemented using the explant of choice. The first generation of mutagenized seeds (defined as M1 population) is typically chimeric; i.e., different cells make different genotypes due to the multicellular stage of embryos in seeds. Hence, M1 plants are self-pollinated to generate M2 population which will be used for DNA isolation. Most mutational events are recessive, as such recessive genes are not detected during the M1 needing further segregation analysis at the M2.

3.1.4. DNA Isolation and Pooling

Tissue from individual M2 plants is used for genomic DNA isolation using either high-throughput 96-well plate procedures or small scale methods. After DNA isolation, the quality and quantity of the DNA needs to be investigated. Once identical DNA concentration is obtained, pooling DNA samples is made to reduce the cost and time of screening.

3.1.5. Primer Design and PCR Amplification

As a reverse-genetics technique, TILLING is used to screen for mutations in specific genes which are expected or known to correspond to the trait of interest. It is important to design a set of primers which are specific to amplify only the gene of interest. Specificity of primer is important especially for members of multi-gene families or in polyploid species where multiple homoeologues genes are present [47, 66, 68]. This is commonly achieved by using copy-specific primers. In polyploid species, specificity can be achieved by designing primers in more divergent regions particularly in the introns or in the 5' and 3' UTR regions [45, 47]. The PCR amplification is followed by the heteroduplex formation step where the products of the PCR are first denatured followed by slow annealing to facilitate the formation of heteroduplex molecules [69, 70].

3.1.6. Mutation Detection

Several single-strand specific nuclease, members of the S1 nuclease family (e.g. CEL I or mung bean nuclease), recognize and cleave the mismatches formed in heteroduplexes [44, 67]. CEL I is the most commonly used and preferred enzyme for mutation detection [44, 63]. CEL I cleaves to the 3' side of mismatches in heteroduplexes while leaving homoduplexes intact; hence two complementary fragments are formed [71]. It is available from commercial suppliers (Surveyor Mutation Detection Kit; Transgenomic®) or extracted from celery stalks [63]. Endonucleases such as Brassica petiole extract [48] and ENDO1 [56] have also proven to be efficient in cleaving heteroduplexes.

Mutation detection after cleavage of the heteroduplex can be done using different methods. The most commonly used method is through a denaturing polyacrylamide gel run on a LI-COR DNA analyzer (referred as LI-COR) system. For this approach, PCR products are amplified using Infra-red dye (IRD) labelled primers. Both, the forward- and reverse-primers are labelled at the 5'-end with a specific dye to be detected in one of the two channels of the LI-COR. After PCR amplification and endonuclease digestion, products are loaded on 5-6% denaturing polyacrylamide gels.

High Resolution Melt (HRM) analysis which depends on the loss of fluorescent from the intercalating dyes bound to
Table 2. Selected TILLING platforms and sources of mutagenized populations for model and crop plants.

| Plant (species)               | TILLING Platform/ project | Host Institution, Country                  | Reference |
|-------------------------------|---------------------------|--------------------------------------------|-----------|
| *Arabidopsis thaliana*        | CAN-TILL                  | Uni. British Columbia, Canada              | [86]      |
|                               | URGV TILLING              | URGV, Versailles, France                   | [75]      |
|                               | UCD TILLING               | Univ. California, Davis, USA               | [87]      |
| *Barley (Hordeum vulgare)*    | Barley TILLING            | SCRI, Scotland, UK                         | [88]      |
|                               | TILLmore (cv. Morex)      | DIISTA, Bologna, Italy                     | [89]      |
| *Field mustard (Brassica rapa)* | B. rapa TILLING           | John Innes, UK                             | [90]      |
|                               | MBGP TILLING              | Multinational Consortia                    | [91]      |
|                               | RevGenUK                  | JIC, Norwich, UK                           | [92]      |
| *Brachypodium distachyon*     | BRACHYTIL                 | URGV, Versailles, France                   | [75]      |
| *Cucumber (Cucumis sativus)*  | URGV TILLING              | URGV, Versailles, France                   | [75]      |
| *Durum wheat (Triticum turgidum)* | Wheat TILLING            | Univ. California, Davis, USA               | [93]      |
| *Flax (Linum usitatissimum)*  | PT-Flax                   | UGSF, France                               | [94]      |
| *Lotus japonicus*             | Lotus TILLING             | LMU, Munich, Germany                       | [95]      |
|                               | RevGenUK                  | John Innes Centre, UK                      | [92]      |
| *Medicago truncatula*         | Medicago TILLING          | CRA, Lodi, Italy                           | [96]      |
|                               | RevGenUK                  | JIC, Norwich, UK                           | [92]      |
| *Melon (Cucumis melo)*        | URGV TILLING              | URGV, Versailles, France                   | [75]      |
| *Pea (Pisum sativum)*         | PETILL                    | URGV, Versailles, France                   | [75]      |
| *Pepper (Capsicum spp.)*      | URGV TILLING              | URGV, Versailles, France                   | [75]      |
| *Rapeseed (Brassica napus)*   | CAN-TILL                  | Uni. British Columbia, Canada              | [86]      |
|                               | MBGP TILLING              | Multinational Consortia                    | [91]      |
| *Rice (Oryza sativa)*         | RICE-TILL (Volano)        | CRA, Lodi, Italy                           | [96]      |
|                               | UCD TILLING               | Univ. California, Davis, USA               | [87]      |
| *Rye (Secale cereale)*        | GABI-TILL Project         | TUM, Munich, Germany                       | [97]      |
| *Tomato (Solanum lycopersicum)* | TOMATILL                 | URGV, Versailles, France                   | [75]      |
|                               | Micro-TOM TILLING         | Univ. Tsukuba, Japan                       | [98]      |
|                               | UCD TILLING               | Univ. California, Davis                    | [87]      |
| *Tef (Eragrostis tef)*        | Tef TILLING               | Univ. Bern, Switzerland                    | [99]      |
| *Watermelon (Citrullus lanatus)* | URGV TILLING             | URGV, Versailles, France                   | [75]      |
| *Wild cabbage (Brassica oleracea)* | CAN-TILL               | Uni. British Columbia                      | [86]      |
|                               | MBGP TILLING              | Multinational Consortia                    | [91]      |

double strand DNA is also effective in mutation detection [46, 72]. Alternative methods such as the agarose electrophoresis and the non-denaturing polyacrylamide gel are affordable in labs with resource limitation [47, 48].

Next Generation Sequencing (NGS) platforms have also been implemented in mutant detection [73, 74]. Some of the benefits of sequence based TILLING approaches are: i) the sequence of each mutation and its impact on protein sequence is directly determined, ii) it does not rely on either labelled primers or endonucleases, iii) it is based on an objective statistical method and not on visual inspections, iv) it is flexible with respect to changing numbers of samples and amplicons, and v) since it is based on highly redundant sequencing, it reduces the likelihood of identifying false positives [73, 74]. Hence, NGS-based mutation detection system
is robust and the most preferred one among other detection methods. The pros and cons as well logistics of six mutation discovery methods were recently reported [62].

3.1.7. Validation of the Mutation by Sequencing and Crossing

Mutations detected by gel-based TILLING methods need to be confirmed by sequencing. If the LI-COR is used, since the detected mutation corresponds to the location of the polymorphism, it makes confirmation by sequencing quite efficient. The labelled primers on the LI-COR provide a directionality (5’- or 3’-end) which allows the sequencing reaction to target the specific site [50, 70]. On the other hand, the alternative screening methods which use unlabeled primers (e.g. agarose electrophoresis and non-denaturing polyacrylamide gels) do not provide an exact position of the mutation. The stable inheritance of the mutation in the gene of interest is confirmed by crossing a mutant line to the original or other lines and investigate the co-segregation of the mutation in the F2 population.

3.2. TILLING Platforms and Their Achievements

Since the first successful TILLING in plants [19], a number of platforms have been established in diverse crops. A list of current TILLING platforms is shown in (Table 2). Among these, the URGV at Versailles in France takes the largest share as it has 18 mutant collections in 8 crop species which include melon, pea, pepper and tomato [75]. The same group has established the online searchable database which contains the phenotypic and sequence information for pea mutant population [76]. Similarly, an open-source bioinformatics tool called LIMSTILL provides support in amplicon selection, primer design, sequence analysis, and annotation of mutations [77].

3.3. Useful Mutants Discovered Through TILLING

The TILLING technique has enabled researchers to identify mutants in the traits of interest. A list of selected crops in which mutations in key agronomic and nutritional-related traits occurred are shown in (Table 3). Among these, the prominent ones are the bread wheat and tomato with enhanced disease resistance and a rice plant with semi-dwarf

| Table 3. Important traits altered in TILLING populations of diverse crops. |
|-------------------------------------------------|--------------------------|---------------------------------|-----------------|
| **Crop**                                          | **Gene Name**           | **Desirable Mutant Phenotype or Trait** | **Reference** |
|---------------------------------------------------|-------------------------|----------------------------------------|---------------|
| **Morphological and agronomic related traits**     |                          |                                        |               |
| Bread wheat (Triticum aestivum)                    | PFT1 (Phytochrome and Flowering Time1) | Disease resistance                  | [78]          |
| Rice (Oryza sativa)                               | SD1 (Semi-dwarf 1)      | Semi-dwarf & lodging tolerant         | [79]          |
| Tomato (Lycopersicon esculentum)                  | cIF4E                   | Virus resistance                       | [80]          |
| **Nutritional and health related traits**          |                          |                                        |               |
| Barley (Hordeum vulgare)                          | GBSSI (Granule-bound starch synthase I) | Reduction in amylose: amyllopectin ratio | [100]        |
| Oats (Avena sativa)                               | AsPAL1 (phenylalanine ammonia-lyase) | Increased digestibility               | [81]          |
| Bread wheat                                       | SBEIIa (Starch branching enzyme IIa) | High amylose & resistant starch       | [101]        |
| Peanut (Arachis hypogaea)                         | GBSSI (Granule-bound starch synthase I) | Near null-waxy                       | [45]          |
| Rapeseed (Brassica napus)                         | Ara h (Arachis hypogaea h). | Allergen reduction                   | [82]          |
| Rice                                              | ITPK (inositol(1,3,4)P3 5/6-kinase) | Low phytic acid                     | [83]          |
| Sorghum (Sorghum bicolor)                         | CYP79A1 (Cytochrome P450 79A1) | acyanogenic                          | [103]        |
| Soybean (Glycine max)                             | COMT (Caffeic O-Methyltranferase) | altered lignin content & increased digestibility | [55] |
| Tomato                                            | FAD3-2a (Fatty acid desaturase) | Low alpha-linolenic acid             | [104]        |
|                                                    | RS2 (Raffinose synthase) & FAD2-1A (Fatty acid desaturase) | increase in oleic acid and decrease in linoleic acid | [105] |
|                                                    | GMP (GDP-D-mannose pyrophosphorylase); GME (GDP-D-mannose 3’,5’ epimerase); GGP (GDP-L-galactose phosphorylase) | Ascorbate deficient | [106] |
|                                                    | SIETR1 (six ethylene receptor 1) & SIETR6 (six ethylene receptor 6) | reduced ethylene response or increased shelf-life | [59] |
stature [78-80]. Many TILLING projects gave emphasis to alter nutrition- and health-related traits so that the consumption of plants for food and feed is enhanced. Major achievements in this area include increased digestibility of oats and sorghum [55, 81], reduced allergy level in peanut [82], low level of phytic acid in rice [83], and increased shelf-life in tomato [59]. Due to improved performance, several crops derived from TILLING have already reached the market. Among these, tomatoes with long shelf-life and wheat with increased total dietary fiber (TDF) are commercialized by the private company called Arcadia [84].

3.4. Challenges in TILLING

Although TILLING is key in identifying allelic mutations for the gene of interest, it cannot target gene families due to high specificity of primers. In addition, TILLING plants with polyploidy genome is difficult if not impossible due to the challenge in designing genome specific primer(s) especially if the homoeologues genes have high homology among themselves [43]. Mutation density is another challenge to the TILLING since low mutation rate affects both the efficiency and cost of the screening.

CONCLUSION

Mutation breeding and TILLING have been playing major role in first introducing the mutation and then identifying and applying these mutations in crop improvement. Key points to be considered while running TILLING are to determine the genotype and part of the plant to be used as an explant, the type and concentration of the mutagen, the size of population to be screened, and mutation discovery technique. Several TILLING platforms are nowadays exist for several model and crop plants. Through this, mutants harboring desirable traits have been identified and being incorporated into breeding programs. Although TILLING is a high-throughput reverse genetics activity which can precisely pinpoint mutations in traits which cannot be easily targeted using forward genetics, mutations in visually recognizable traits such as plant height can be investigated using the forward genetics approach. In general, TILLING has become popular since it can effectively introduce mutation or variability in crop plants and harness this variability to improve crop plants. Once established, the TILLING population can be used in screening for several traits of interest.

LIST OF ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| GBS          | Genotyping-By-Sequencing |
| GBSSI        | Granule-bound Starch Synthase I |
| GME          | GDP-D-mannose 3’,5’ epimerase |
| GMP          | GDP-D-mannose Pyrophosphorylase |
| GWAS         | Genome Wide Association Studies |
| HRM          | High Resolution Melting |
| INDELs       | Insertion and Deletions |
| IRD          | Infra-red dye |
| MAS          | Marker-Assisted Selection |
| MNU          | N-methyl-N-nitrosourea |
| MVD          | Mutant Variety Database |
| NaN3         | Sodium azide |
| NO           | Nitric oxide |
| PFT1         | Phytochrome and Flowering Timel |
| RAD          | Restriction-site Associated DNA |
| RNAi         | RNA Interference |
| RS2          | Raffinose synthase 2 |
| SBEIIa       | Starch Branching Enzyme IIa |
| SD1          | Semi-dwarf 1 |
| SIETR        | Six Ethylene Receptor |
| SNP          | Single Nucleotide Polymorphism |
| SSR          | Simple Sequence Repeat, or microsatellite |
| TAIL-PCR     | Thermal Asymmetric Interlaced PCR |
| TALEN        | Transcription Activator-Like Effector Nuclease |
| TDF          | Total Dietary Fiber |
| TILLING      | Targeting Induced Local Lesion IN Genomes |
| ZFN          | Zinc-Finger Nuclease |

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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