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REVIEW ARTICLE

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

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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-

Table 1. Types of natural and induced mutations or polymorphisms and their detection methods.

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, NaN_3, 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 (Genome editing)	Meganuclease	DSB & repair			[21]
	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 Deleteagene 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) *splice 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 biosafety 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-dwarfism 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 F_2 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 population from which pollen was mutagenized by EMS [44]. Parts of plants were also successfully used for mutagenesis especially for vegetatively propagated plants. Nodal segments containing 1-2 buds from potato [64] and shoot tips from banana [65] have been used as an explant. 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].

3.1.2. 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 mainly detects nucleotide polymorphisms, mutagens which create point mutation are preferentially selected to generate TILLING populations. A mutagen proved to induce point mutation and widely used in developing TILLING populations is EMS [45, 52, 55]. EMS normally creates G:C to A:T transitions in the genome due to the alkylation of G nucleotide residues which then pairs with T instead of C [67]. Mutations in the coding region of the gene might alter plant metabolism or the effective level of a gene product that might be useful for breeding. Prior to introducing large-scale mutagenesis, pilot studies should be made to determine the right type and concentration of the mutagen.

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 M_1 population) is typically chimeric; i.e., different cells make different genotypes due to the multicellular stage of embryos in seeds. Hence, M_1 plants are self-pollinated to generate M_2 population which will be used for DNA isolation. Most mutational events are recessive, as such recessive genes are not detected during the M_1 needing further segregation analysis at the M_2.

3.1.4. DNA Isolation and Pooling

Tissue from individual M_2 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 nucleases, 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)	DiSTA, 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, i 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 F_2 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*)	eIF4E	Virus resistance	[80]
Nutritional and health related traits			
Barley (*Hordeum vulgare*)	GBSSI (Granule-bound starch synthase I)	Reduction in amylose: amylopectin ratio	[100]
Oats (*Avena sativa*)	AsPAL1 (phenylalanine ammonia-lyase)	Increased digestibility	[81]
Bread wheat	SBEIIa (Starch branching enzyme IIa)	High amylose & resistant starch	[101]
	GBSSI (Granule-bound starch synthase I)	Near null-waxy	[45]
Peanut (*Arachis hypogaea*)	Ara h (*Arachis hypogaea* h).	Allergen reduction	[82]
Rapeseed (*Brassica napus*)	Bnax (*Brassica napus* X)	Reduction in sinapine	[102]
Rice	ITPK (inositol(1,3,4)P3 5/6-kinase)	Low phytic acid	[83]
Sorghum (*Sorghum bicolor*)	CYP79A1 (Cytochrome P450 79A1)	acyanogenic	[103]
	COMT (Caffeic O-Methyltranferase)	altered lignin content & increased digestibility	[55]
Soybean (*Glycine max*)	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]
Tomato	GMP (GDP-D-mannose pyrophosphorylase); GME (GDP-D-mannose 3',5' epimerase); GGP (GDP-L-galactose phosphorylase)	Ascorbate deficient	[106]
	SlETR1 (six ethylene receptor 1) & SlETR6 (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 low 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 homoelogues 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

AFLP	=	Amplified Fragment Length Polymorphism
CRISPR/Cas9	=	Clustered, Regularly Interspaced, Short Palindromic Repeat/ CRISPR-Associated 9
Deleteagene	=	Delete-a-gene
EMS	=	Ethyl-Methane Sulfonate
ENU	=	N-ethyl-N-Nitrosourea
FAD	=	Fatty Acid Desaturase
FAO/IAEA	=	Food and Agriculture Organization/International Atomic Energy Agency
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
NaN_3	=	Sodium azide
NO	=	Nitric oxide
PFT1	=	Phytochrome and Flowering Time1
RAD	=	Restriction-site Associated DNA
RNAi	=	RNA Interference
RS2	=	Raffinose synthase 2
SBEIIa	=	Starch Branching Enzyme IIa
SD1	=	Semi-dwarf 1
SlETR	=	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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REFERENCES

[1] Maqbool, S. B.; Devi, P.; Sticklen, M. B. Biotechnology: Genetic improvement of sorghum (Sorghum bicolor (L.) Moench). *In Vitro Cell Dev-Pl.,* **2001**, *37* (5), 504-515.

[2] Vasil, I. K. Plant-Tissue Culture and Molecular-Biology as Tools in Understanding Plant Development and in Plant Improvement. *Curr. Opin. Biotechnol.,* **1991**, *2* (2), 158-163.

[3] Lakshmanan, P.; Taji, A. Somatic embryogenesis in leguminous plants. *Plant Biol.,* **2000**, *2* (2), 136-148.

[4] Bal, U.; Abak, K. Haploidy in tomato (Lycopersicon esculentum Mill.): a critical review. *Euphytica,* **2007**, *158* (1-2), 1-9.

[5] Ochoa-Alejo, N.; Ramirez-Malagon, R. Invited review: *In vitro* chili pepper biotechnology. *In Vitro Cell Dev-Pl.,* **2001**, *37* (6), 701-729.

[6] Wahid, M. B.; Abdullah, S. N. A.; Henson, I. E. Oil palm - Achievements and potential. *Plant Prod. Sci.,* **2005**, *8* (3), 288-297.

[7] Uma, S.; Lakshmi, S.; Saraswathi, M. S.; Akbar, A.; Mustaffa, M. M. Embryo rescue and plant regeneration in banana (Musa spp.). *Plant Cell Tiss. Org.,* **2011**, *105* (1), 105-111.

[8] Giri, C. C.; Shyamkumar, B.; Anjaneyulu, C. Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. *Trees-Struct. Funct.,* **2004**, *18* (2), 115-135.

[9] Golle, D. P.; Reiniger, L. R. S.; Curti, A. R.; Bevilacqua, C. B. Forestry improvement: emphasis on biotechnology application. *Cienc. Rural,* **2009**, *39* (5), 1606-1613.

[10] Charcosset, A.; Moreau, L. Use of molecular markers for the development of new cultivars and the evaluation of genetic diversity. *Euphytica,* **2004**, *137* (1), 81-94.

[11] James, C. Global Status of Commercialized Biotech/GM Crops: 2014. ISAAA Brief No. 49; Ithaca, NY, 2014.

[12] IAEA FAO/IAEA Programme's Database of Mutation Enhanced Technologies for Agriculture (META). http://mvgs.iaea.org/ (accessed June 29, 2015).

[13] IAEA/FAO Mutation Induction. http://www-naweb.iaea.org/nafa/pbg/mutation-induction.html (accessed June 29, 2015).

[14] Xiong, J.-S.; Ding, J.; Li, Y. Genome-editing technologies and their potential application in horticultural crop breeding. *Horticulture Res.,* **2015**, *2*, 15019.

[15] Sikora, P.; Chawade, A.; Larsson, M.; Olsson, J.; O. Mutagenesis as a Tool in Plant Genetics, Functional Genomics, and Breeding. *Int. J. Plant Genomics,* **2011**, *2011*, 314829.

[16] Mba, C. Induced Mutations Unleash the Potentials of Plant Genetic Resources for Food and Agriculture. *Agronomy,* **2013**, *3* (1), 200-231.

[17] Li, X.; Lassner, M.; Zhang, Y. L. Deleteagene: a fast neutron deletion mutagenesis-based gene knockout system for plants. *Comp. Funct. Genomics,* **2002**, *3* (2), 158-160.

[18] Chen, J. M. Genomic Rearrangements: Mutational Mechanisms. In *Encyclopedia of Life Sciences (ELS)*, John Wiley & Sons, Ltd: Chichester: 2011.

[19] McCallum, C. M.; Comai, L.; Greene, E. A.; Henikoff, S. Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiol.,* **2000**, *123* (2), 439-42.

[20] Kurowska, M.; Daszkowska-Golec, A.; Gruszka, D.; Marzec, M.; Szurman, M.; Szarejko, I.; Maluszynski, M. TILLING - a shortcut in functional genomics. *J. Appl. Genet.,* **2011**, *52* (4), 371-390.

[21] Smith, J.; Grizot, S.; Arnould, S.; Duclert, A.; Epinat, J. C.; Chames, P.; Prieto, J.; Redondo, P.; Blanco, F. J.; Bravo, J.; Montoya, G.; Paques, F.; Duchateau, P. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. *Nucleic Acids Res.,* **2006**, *34* (22).

[22] Shukla, V. K.; Doyon, Y.; Miller, J. C.; DeKelver, R. C.; Moehle, E. A.; Worden, S. E.; Mitchell, J. C.; Arnold, N. L.; Gopalan, S.; Meng, X.; Choi, V. M.; Rock, J. M.; Wu, Y. Y.; Katibah, G. E.; Zhifang, G.; McCaskill, D.; Simpson, M. A.; Blakeslee, B.; Greenwalt, S. A.; Butler, H. J.; Hinkley, S. J.; Zhang, L.; Rebar, E. J.; Gregory, P. D.; Urnov, F. D. Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature,* **2009**, *459* (7245), 437-41.

[23] Townsend, J. A.; Wright, D. A.; Winfrey, R. J.; Fu, F.; Maeder, M. L.; Joung, J. K.; Voytas, D. F. High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature,* **2009**, *459* (7245), 442-5.

[24] Cermak, T.; Doyle, E. L.; Christian, M.; Wang, L.; Zhang, Y.; Schmidt, C.; Baller, J. A.; Somia, N. V.; Bogdanove, A. J.; Voytas, D. F. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting (vol 39, pg e82, 2011). *Nucleic Acids Res.,* **2011**, *39* (17), 7879-7879.

[25] Li, T.; Liu, B.; Spalding, M. H.; Weeks, D. P.; Yang, B. High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat Biotechnol* **2012**, *30* (5), 390-392.

[26] Nekrasov, V.; Staskawicz, B.; Weigel, D.; Jones, J. D. G.; Kamoun, S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.,* **2013**, *31* (8), 691-693.

[27] Li, J. F.; Norville, J. E.; Aach, J.; McCormack, M.; Zhang, D. D.; Bush, J.; Church, G. M.; Sheen, J. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat. Biotechnol.,* **2013**, *31* (8), 688-691.

[28] Shan, Q. W.; Wang, Y. P.; Li, J.; Zhang, Y.; Chen, K. L.; Liang, Z.; Zhang, K.; Liu, J. X.; Xi, J. J.; Qiu, J. L.; Gao, C. X. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.,* **2013**, *31* (8), 686-688.

[29] Jones, H. D. Regulatory uncertainty over genome editing. *Nat. Plants,* **2015**, *1*, 14011.

[30] Araki, M.; Ishii, T. Towards social acceptance of plant breeding by genome editing. *Trends Plant Sci.,* **2015**, *20* (3), 145-149.

[31] Bortesi, L.; Fischer, R. The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv.,* **2015**, *33* (1), 41-52.

[32] AGI CRISPR-PLANT: A Portal of CRISPR-Cas9 Mediated Genome Editing. http://www.genome.arizona.edu/crispr/ (accessed June 29, 2015).

[33] Lei, Y.; Lu, L.; Liu, H. Y.; Li, S.; Xing, F.; Chen, L. L. CRISPR-P: A Web Tool for Synthetic Single-Guide RNA Design of CRISPR-System in Plants. *Mol. Plant,* **2014**, *7* (9), 1494-1496.

[34] CRISPR-P A web tool for synthetic single-guide RNA design of CRISPR-system in plants. http://cbi.hzau.edu.cn/crispr/ (accessed June 29, 2015).

[35] Anonymous Amplified Fragment Length Polymorphism (AFLP®) Analysis on Applied Biosystems Capillary Electrophoresis Systems; **2005**; p 4.

[36] Miah, G.; Rafii, M. Y.; Ismail, M. R.; Puteh, A. B.; Rahim, H. A.; Islam, K. N.; Latif, M. A. A Review of Microsatellite Markers and Their Applications in Rice Breeding Programs to Improve Blast Disease Resistance. *Int. J. Mol. Sci.,* **2013**, *14* (11), 22499-22528.

[37] Elshire, R. J.; Glaubitz, J. C.; Sun, Q.; Poland, J. A.; Kawamoto, K.; Buckler, E. S.; Mitchell, S. E. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *Plos One,* **2011**, *6* (5), e19379.

[38] Baird, N. A.; Etter, P. D.; Atwood, T. S.; Currey, M. C.; Shiver, A. L.; Lewis, Z. A.; Selker, E. U.; Cresko, W. A.; Johnson, E. A. Rapid SNP Discovery and Genetic Mapping Using Sequenced RAD Markers. *Plos One* **2008**, *3* (10).

[39] Comai, L.; Young, K.; Till, B. J.; Reynolds, S. H.; Greene, E. A.; Codomo, C. A.; Enns, L. C.; Johnson, J. E.; Burtner, C.; Odden, A. R.; Henikoff, S. Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. *Plant J.,* **2004**, *37* (5), 778-786.

[40] Abe, A.; Kosugi, S.; Yoshida, K.; Natsume, S.; Takagi, H.; Kanzaki, H.; Matsumura, H.; Mitsuoka, C.; Tamiru, M.; Innan, H.; Cano, L.; Kamoun, S.; Terauchi, R. Genome sequencing reveals agronomically important loci in rice using MutMap. *Nat. Biotechnol.,* **2012**, *30* (2), 174-8.

[41] Liu, Y. G.; Whittier, R. F. Thermal Asymmetric Interlaced Pcr - Automatable Amplification and Sequencing of Insert End Fragments from P1 and Yac Clones for Chromosome Walking. *Genomics,* **1995**, *25* (3), 674-681.

[42] Alonso, J. M.; Ecker, J. R. Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in Arabidopsis. *Nat. Rev. Genetics,* **2006**, *7*, 524-536.

[43] Tadele, Z.; Mba, C.; Till, B. J. TILLING for mutations in model plants and crops. In *Molecular Techniques in Crop Improvement: 2nd Edition* Jain, S. M.; Brar, D. S., Eds. Springer: 2010; pp 307-332.

[44] Till, B. J.; Reynolds, S. H.; Weil, C.; Springer, N.; Burtner, C.; Young, K.; Bowers, E.; Codomo, C. A.; Enns, L. C.; Odden, A. R.; Greene, E. A.; Comai, L.; Henikoff, S. Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biol.,* **2004**, *4*, 12.

[45] Slade, A. J.; Fuerstenberg, S. I.; Loeffler, D.; Steine, M. N.; Facciotti, D. A reverse genetic, nontransgenic approach to wheat crop

improvement by TILLING. *Nat. Biotechnol.,* **2005**, *23* (1), 75-81.

[46] Dong, C. M.; Vincent, K.; Sharp, P. Simultaneous mutation detection of three homoeologous genes in wheat by High Resolution Melting analysis and Mutation Surveyor (R). *BMC Plant Biol.,* **2009**, *9*.

[47] Uauy, C.; Paraiso, F.; Colasuonno, P.; Tran, R. K.; Tsai, H.; Berardi, S.; Comai, L.; Dubcovsky, J. A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biol.,* **2009**, *9*, 115.

[48] Sato, Y.; Shirasawa, K.; Takahashi, Y.; Nishimura, M.; Nishio, T. Mutant selection from progeny of gamma-ray-irradiated rice by DNA heteroduplex cleavage using Brassica petiole extract. *Breeding Sci.,* **2006**, *56* (2), 179-183.

[49] Raghavan, C.; Naredo, M. E. B.; Wang, H. H.; Atienza, G.; Liu, B.; Qiu, F. L.; McNally, K. L.; Leung, H. Rapid method for detecting SNPs on agarose gels and its application in candidate gene mapping. *Mol. Breeding,* **2007**, *19* (2), 87-101.

[50] Till, B. J.; Cooper, J.; Tai, T. H.; Colowit, P.; Greene, E. A.; Henikoff, S.; Comai, L. Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biol.,* **2007**, *7*, 19.

[51] Suzuki, T.; Eiguchi, M.; Kumamaru, T.; Satoh, H.; Matsusaka, H.; Moriguchi, K.; Nagato, Y.; Kurata, N. MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice. *Mol. Genet. Genomics,* **2008**, *279* (3), 213-223.

[52] Caldwell, D. G.; McCallum, N.; Shaw, P.; Muehlbauer, G. J.; Marshall, D. F.; Waugh, R. A structured mutant population for forward and reverse genetics in Barley (Hordeum vulgare L.). *Plant j. Cell Mol. Biol.,* **2004**, *40* (1), 143-50.

[53] Talame, V.; Bovina, R.; Sanguineti, M. C.; Tuberosa, R.; Lundqvist, U.; Salvi, S. TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotechnol. J.,* **2008**, *6* (5), 477-485.

[54] Lababidi, S.; Mejlhede, N.; Rasmussen, S. K.; Backes, G.; Al-Said, W.; Baum, M.; Jahoor, A. Identification of barley mutants in the cultivar 'Lux' at the Dhn loci through TILLING. *Plant Breeding,* **2009**, *128* (4), 332-336.

[55] Xin, Z. G.; Wang, M. L.; Barkley, N. A.; Burow, G.; Franks, C.; Pederson, G.; Burke, J. Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biol.,* **2008**, *8*.

[56] Triques, K.; Sturbois, B.; Gallais, S.; Dalmais, M.; Chauvin, S.; Clepet, C.; Aubourg, S.; Rameau, C.; Caboche, M.; Bendahmane, A. Characterization of Arabidopsis thaliana mismatch specific endonucleases: application to mutation discovery by TILLING in pea. *Plant J.,* **2007**, *51* (6), 1116-1125.

[57] Elias, R.; Till, B. J.; Mba, C.; Al-Safadi, B. Optimizing TILLING and Ecotilling techniques for potato (Solanum tuberosum L). *BMC Res. Notes,* **2009**, *2*, 141.

[58] Minoia, S.; Petrozza, A.; D'Onofri, O.; Piron, F.; Mosca, G.; Sozio, G.; Cellini, F.; Bendahmane, A.; Carriero, F. A new mutant genetic resource for tomato crop improvement by TILLING technology. *BMC Res. Notes,* **2010**, *3*, 69.

[59] Okabe, Y.; Asamizu, E.; Saito, T.; Matsukura, C.; Ariizumi, T.; Bres, C.; Rothan, C.; Mizoguchi, T.; Ezura, H. Tomato TILLING Technology: Development of a Reverse Genetics Tool for the Efficient Isolation of Mutants from Micro-Tom Mutant Libraries. *Plant Cell Physiol.,* **2011**, *52* (11), 1994-2005.

[60] Cooper, J. L.; Till, B. J.; Laport, R. G.; Darlow, M. C.; Kleffner, J. M.; Jamai, A.; El-Mellouki, T.; Liu, S.; Ritchie, R.; Nielsen, N.; Bilyeu, K. D.; Meksem, K.; Comai, L.; Henikoff, S. TILLING to detect induced mutations in soybean. *BMC Plant Biol.,* **2008**, *8*.

[61] Gilchrist, E. J.; Sidebottom, C. H. D.; Koh, C. S.; MacInnes, T.; Sharpe, A. G.; Haughn, G. W. A Mutant Brassica napus (Canola) Population for the Identification of New Genetic Diversity via TILLING and Next Generation Sequencing. *Plos One,* **2013**, *8* (12), e84303.

[62] Chen, L.; Hao, L. G.; Parry, M. A. J.; Phillips, A. L.; Hu, Y. G. Progress in TILLING as a tool for functional genomics and improvement of crops. *J. Integr. Plant Biol.,* **2014**, *56* (5), 425-443.

[63] Till, B. J.; Zerr, T.; Comai, L.; Henikoff, S. A protocol for TILLING and Ecotilling in plants and animals. *Nat. Protoc.,* **2006**, *1* (5), 2465-77.

[64] Al-Safadi, B.; Arabi, M. I. E. *In vitro* induction, isolation, and selection of potato mutants tolerant to salinity. *Adv. Horticultural Sci.,* **2007**, *21* (3), 127-132.

[65] Jankowicz-Cieslak, J.; Huynh, O. A.; Brozynska, M.; Nakitandwe, J.; Till, B. J. Induction, rapid fixation and retention of mutations in vegetatively propagated banana. *Plant Biotechnol. J.,* **2012**, *10* (9), 1056-1066.

[66] Slade, A. J.; Knauf, V. C. TILLING moves beyond functional genomics into crop improvement. *Transgenic Res.,* **2005**, *14* (2), 109-15.

[67] Comai, L.; Henikoff, S. TILLING: practical single-nucleotide mutation discovery. *Plant J.,* **2006**, *45* (4), 684-94.

[68] Esfeld, K.; Uauy, C.; Tadele, Z. Application of TILLING for Orphan Crop Improvement In *Biotechnology of Neglected and Underutilized Crops*, Jain, S. M.; Gupta, S. D., Eds. Springer: **2013**; pp 83-113.

[69] Till, B. J.; Reynolds, S. H.; Greene, E. A.; Codomo, C. A.; Enns, L. C.; Johnson, J. E.; Burtner, C.; Odden, A. R.; Young, K.; Taylor, N. E.; Henikoff, J. G.; Comai, L.; Henikoff, S. Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Res.,* **2003**, *13* (3), 524-30.

[70] Gilchrist, E. J.; Haughn, G. W. TILLING without a plough: a new method with applications for reverse genetics. *Curr. Opin. Plant Biol.,* **2005**, *8* (2), 211-5.

[71] Henikoff, S.; Till, B. J.; Comai, L. TILLING. Traditional mutagenesis meets functional genomics. *Plant Physiol.,* **2004**, *135* (2), 630-6.

[72] Lochlainn, S. O.; Amoah, S.; Graham, N. S.; Alamer, K.; Rios, J. J.; Kurup, S.; Stoute, A.; Hammond, J. P.; Ostergaard, L.; King, G. J.; White, P. J.; Broadley, M. R. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. *Plant Methods,* **2011**, *7*.

[73] Rigola, D.; van Oeveren, J.; Janssen, A.; Bonne, A.; Schneiders, H.; van der Poel, H. J.; van Orsouw, N. J.; Hogers, R. C.; de Both, M. T.; van Eijk, M. J. High-throughput detection of induced mutations and natural variation using KeyPoint technology. *Plos One,* **2009**, *4* (3), e4761.

[74] Tsai, H.; Howell, T.; Nitcher, R.; Missirian, V.; Watson, B.; Ngo, K. J.; Lieberman, M.; Fass, J.; Uauy, C.; Tran, R. K.; Khan, A. A.; Filkov, V.; Tai, T. H.; Dubcovsky, J.; Comai, L. Discovery of rare mutations in populations: TILLING by sequencing. *Plant Physiol.,* **2011**, *156* (3), 1257-68.

[75] URGV TILLING Platform. http://www-urgv.versailles.inra.fr/tilling/index.htm (accessed June 24, 2015).

[76] Dalmais, M.; Schmidt, J.; Le Signor, C.; Moussy, F.; Burstin, J.; Savois, V.; Aubert, G.; Brunaud, V.; de Oliveira, Y.; Guichard, C.; Thompson, R.; Bendahmane, A. UTILLdb, a Pisum sativum *in silico* forward and reverse genetics tool. *Genome Biol.,* **2008**, *9* (2), R43.

[77] LIMSTILL For Identification of mutations by sequencing and TILLING. http://limstill.niob.knaw.nl/ (accessed July 1, 2015).

[78] Fitzgerald, T. L.; Kazan, K.; Li, Z. Y.; Morell, M. K.; Manners, J. M., A high-throughput method for the detection of homoeologous gene deletions in hexaploid wheat. *BMC Plant Biol.,* **2010**, *10*.

[79] Casella, L.; Greco, R.; Bruschi, G.; Wozniak, B.; Dreni, L.; Kater, M.; Cavigiolo, S.; Lupotto, E.; Piffanelli, P. TILLING in European Rice: Hunting Mutations for Crop Improvement. *Crop Sci.,* **2013**, *53* (6), 2550-2562.

[80] Piron, F.; Nicolai, M.; Minoia, S.; Piednoir, E.; Moretti, A.; Salgues, A.; Zamir, D.; Caranta, C.; Bendahmane, A. An induced mutation in tomato eIF4E leads to immunity to two potyviruses. *Plos One,* **2010**, *5* (6), e11313.

[81] Chawade, A.; Sikora, P.; Brautigam, M.; Larsson, M.; Vivekanand, V.; Nakash, M. A.; Chen, T. S.; Olsson, O. Development and characterization of an oat TILLING-population and identification of mutations in lignin and beta-glucan biosynthesis genes. *BMC Plant Biol.,* **2010**, *10*.

[82] Knoll, J. E.; Ramos, M. L.; Zeng, Y. J.; Holbrook, C. C.; Chow, M.; Chen, S. X.; Maleki, S.; Bhattacharya, A.; Ozias-Akins, P. TILLING for allergen reduction and improvement of quality traits in peanut (Arachis hypogaea L.). *BMC Plant Biol.,* **2011**, *11*.

[83] Kim, S. I.; Tai, T. H. Identification of novel rice low phytic acid mutations via TILLING by sequencing. *Mol. Breeding,* **2014**, *34* (4), 1717-1729.

[84] Arcadia Aracdia Bioscience. http://www.arcadiabio.com/about (accessed June 30, 2015).

[85] Zalapa, J. E.; Cuevas, H.; Zhu, H. Y.; Steffan, S.; Senalik, D.; Zeldin, E.; McCown, B.; Harbut, R.; Simon, P. Using Next-Generation Sequencing Approaches to Isolate Simple Sequence Repeat (Ssr) Loci in the Plant Sciences. *Am. J. Bot.,* **2012**, *99* (2),

193-208.

[86] Can-TILL CAN-TILL: The Canadian TILLING Initiative University of British Columbia Vancouver, B.C., Canada http://www3.botany.ubc.ca/can-till/ (accessed June 24, 2015).

[87] UCD-TILLING Genome TILLING Projects. http://tilling.ucdavis.edu/index.php/Main_Page (accessed June 24, 2015).

[88] Barley-TILLING http://www.scri.ac.uk/research/genetics/platformtechnologies/barleytilling (accessed June 24, 2015).

[89] TIILMore TILLMore: a TILLING resource in Barley Morex. http://www.agrsci.unibo.it/~salvi/tillmore/index.htm (accessed June 24, 2015).

[90] B-rapa https://www.jic.ac.uk/staff/Lars-Ostergaard/tilling.htm (accessed June 24, 2015).

[91] MBGP-TILLING MBGP TILLING consortium. http://www.brassica.info/research/activities/tilling.php (accessed June 24, 2015).

[92] RevGen http://revgenuk.jic.ac.uk/TILLING.htm (accessed June 24, 2015).

[93] Wheat-TILLING http://dubcovskylab.ucdavis.edu/wheat-tilling (accessed June 24, 2015).

[94] PT-FLAX Phenotyping and TILLinG of Flax EMS Mutants. http://flaxomics.univ-lille1.fr/Flaxomics/PTFLAX.html (accessed June 24, 2015).

[95] Lotus-TILLING http://www.genetik.bio.lmu.de/research/parniske/models/lotustilling/index.html (accessed June 24, 2015).

[96] TILLING http://www.tecnoparco.org/index.php?option=com_content&view=article&id=195:tilling&catid=105&Itemid=192&lang=it (accessed June 24, 2015).

[97] GABI-TILL http://plantbreeding.wzw.tum.de/index.php?id=28&L=1 (accessed June 24, 2015).

[98] TOMATOMA Micro-Tom TILLING http://tomatoma.nbrp.jp/ (accessed June 24, 2015).

[99] Tef-TILLING http://www.tef-research.org/ (accessed June 24, 2015).

[100] Sparla, F.; Falini, G.; Botticella, E.; Pirone, C.; Talame, V.; Bovina, R.; Salvi, S.; Tuberosa, R.; Sestili, F.; Trost, P. New Starch Phenotypes Produced by TILLING in Barley. *Plos One,* **2014**, *9* (10).

[101] Slade, A. J.; McGuire, C.; Loeffler, D.; Mullenberg, J.; Skinner, W.; Fazio, G.; Holm, A.; Brandt, K. M.; Steine, M. N.; Goodstal, J. F.; Knauf, V. C. Development of high amylose wheat through TILLING. *BMC Plant Biol.,* **2012**, *12* (1), 69.

[102] Harloff, H. J.; Lemcke, S.; Mittasch, J.; Frolov, A.; Wu, J. G.; Dreyer, F.; Leckband, G.; Jung, C. A mutation screening platform for rapeseed (Brassica napus L.) and the detection of sinapine biosynthesis mutants. *Theor. Appl. Genet.,* **2012**, *124* (5), 957-969.

[103] Blomstedt, C. K.; Gleadow, R. M.; O'Donnell, N.; Naur, P.; Jensen, K.; Laursen, T.; Olsen, C. E.; Stuart, P.; Hamill, J. D.; Moller, B. L.; Neale, A. D. A combined biochemical screen and TILLING approach identifies mutations in Sorghum bicolor L. Moench resulting in acyanogenic forage production. *Plant Biotechnol. J.,* **2012**, *10* (1), 54-66.

[104] Hoshino, T.; Watanabe, S.; Takagi, Y.; Anai, T. A novel GmFAD3-2a mutant allele developed through TILLING reduces alpha-linolenic acid content in soybean seed oil. *Breeding Sci.,* **2014**, *64* (4), 371-377.

[105] Dierking, E. C.; Bilyeu, K. D. New sources of soybean seed meal and oil composition traits identified through TILLING. *BMC Plant Biol.,* **2009**, *9*.

[106] Baldet, P.; Bres, C.; Okabe, Y.; Mauxion, J. P.; Just, D.; Bournonville, C.; Ferrand, C.; Mori, K.; Ezura, H.; Rothan, C. Investigating the role of vitamin C in tomato through TILLING identification of ascorbate-deficient tomato mutants. *Plant Biotechnol.,* **2013**, *30* (3), 309-314.