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TILLING: The Next Generation

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Abstract Gene space: the final frontier in plant functional genomics. These are the voyages of TILLING, the reverse-genetics strategy that sought to boldly go where no-one had gone before by combining high-density chemical mutagenesis with high-throughput mutation discovery. Its 18-year mission has been to explore new technologies such as next generation sequencing and to seek out new strategies like in silico databases of catalogued EMS-induced mutations from entire mutant plant populations. This chapter is a clip show highlighting key milestones in the development of TILLING. Use of different technologies for the discovery of induced mutations, establishment of TILLING in different plant species, what has been learned about the effect of chemical mutagens on the plant genome, development of exome capture sequencing in wheat, and a look to the future of reverse-genetics with targeted genome editing are discussed.

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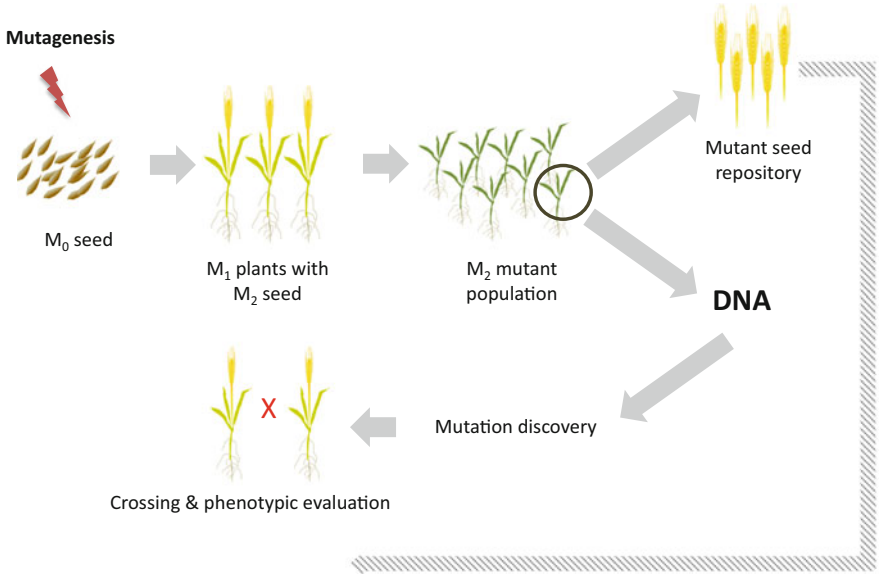
Graphical Abstract



Keywords Chemical mutagenesis, CRISPR/Cas, EMS, In silico TILLING, Next generation sequencing

Contents

Symbol

φ Greek letter Phi

Abbreviations

2X Diploid
4X Tetraploid

6X	Hexaploid
ATP	Arabidopsis TILLING Project
Az	Azide
CIAT	International Center for Tropical Agriculture
CRISPR	Clustered Regularly-Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activator
CRISPRi	CRISPR interference
DNA	Deoxyribonucleic acid
DSBs	Double strand breaks
EMC	Enzymatic mismatch cleavage
EMCA	EMC with aragose gel
EMCC	EMC with capillary electrophoresis
EMCH	EMC with HPLC
EMCL	EMC with LI-COR gels
EMCP	EMC with polyacrylamide gels
EMS	Ethyl methanesulfonate
ENU	*N*-ethyl-*N*-nitrosourea
Gb	Giga bases
HDR	Homology-directed repair
HPLC	High performance liquid chromatography
HRM	High resolution melt
indel	Insertion or deletion of bases
kb	Kilobases
M_0	Plant generation prior to mutagenesis
M_1	First generation of mutagenized plant
M_2	Second generation of mutagenized plant
Mbp	Million base pairs
MNU	*N*-Nitroso-*N*-methylurea
NHEJ	Non-homologous end joining
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
sgRNA	Single guide RNA
SNP	Single nucleotide polymorphism
TALENs	Transcription Activator-Like Effector-based Nucleases
TILLING	Targeting Induced Local Lesions IN Genomes
ZFNs	Zinc finger nucleases

1 Introduction

The Dutch botanist Hugo de Vries is credited as the first person to introduce the word mutation to the scientific vocabulary. His "mutation theory" was based in part on observations of spontaneous and heritable phenotypic changes (mutations) occurring

in evening primroses over a 13-year period [1]. What de Vries was observing was later determined to be the result of large chromosomal aberrations unique to *Oenothera* species. It was the work of Thomas Hunt Morgan and colleagues in the first quarter of the twentieth century on *Drosophila melanogaster* that would popularize the use of the word "mutation" to describe genetic variations in single genes [2]. In addition to stimulating mutation research, de Vries would later go on to describe the phenomenon of genetic recombination in 1903 [3]. Thus, by the early 1900s the major driving forces of genetic diversity, mutation and recombination, were described. These two events underlie biological evolution and provide the means for humans to generate novel diversity in plants and animals (Fig. 1).

Mutations are a particularly useful tool for both geneticist and breeder. New mutations create novel alleles that can have a profound impact on organismal phenotype, and provide the raw material for breeders to create combinations of alleles to improve crop performance [5]. While spontaneous mutations are a major source of heritable phenotypic diversity, they pose a problem for the researcher: they happen quite rarely. Indeed, recent studies employing whole genome sequencing suggest a spontaneous mutation rate of 7.4×10^{-9} in rice and 7×10^{-9} in Arabidopsis [6, 7]. A major milestone, therefore, was the discovery that mutations could be induced much faster than they appear in nature.

Herman Muller used X-rays to create mutations in *Drosophila melanogaster* that accumulated orders of magnitude faster than what was observed spontaneously [8]. Contemporary with this, Lewis John Stadler used X-rays to induce mutations in cereals [9, 10]. The idea that mutations could be used for breeding was quickly adopted and by the late 1930s the first mutant crop variety was released, a cultivar of tobacco named Chlorina that had improved characteristics for cigar smoking [11, 12]. This ushered in the field of plant mutation breeding that has resulted in the official release of more than 3,200 mutant crop varieties [13, 14]. Forward genetic approaches that utilize induced mutations remain popular likely because of the ease of mutation induction in many crops and the fact that phenotypes can be observed without any prior knowledge of genes or gene function.

Activities to determine the sequence of DNA, and thus genes, in organisms began in the 1960s and led to the first full DNA genome (bacteriophage φX) in 1977 [15]. Years later, the development of next-generation sequencing technologies has led to a massive increase in the acquisition of gene sequences that had vastly outpaced the establishment of in vivo functions of genes through direct experimental evidence. Reverse-genetic methods can bridge this gap as they provide direct in vivo testing of the function of genes. The process involves the creation of gene disruptions in the selected genotype, the identification of individuals having affected gene sequences or gene expression, and the testing of these organisms to determine the phenotypic consequence of the mutation (Fig. 2). This is in essence the opposite direction of traditional genetic analysis, where plants are selected based on phenotype and only later are analyzed to determine the genetic alteration that is causative for the observed trait. Thus, the process is the "reverse" of traditional genetic analysis. A key component of reverse-genetic approaches is that they are hypothesis driven endeavors where the researcher seeks to study the in vivo function of a gene

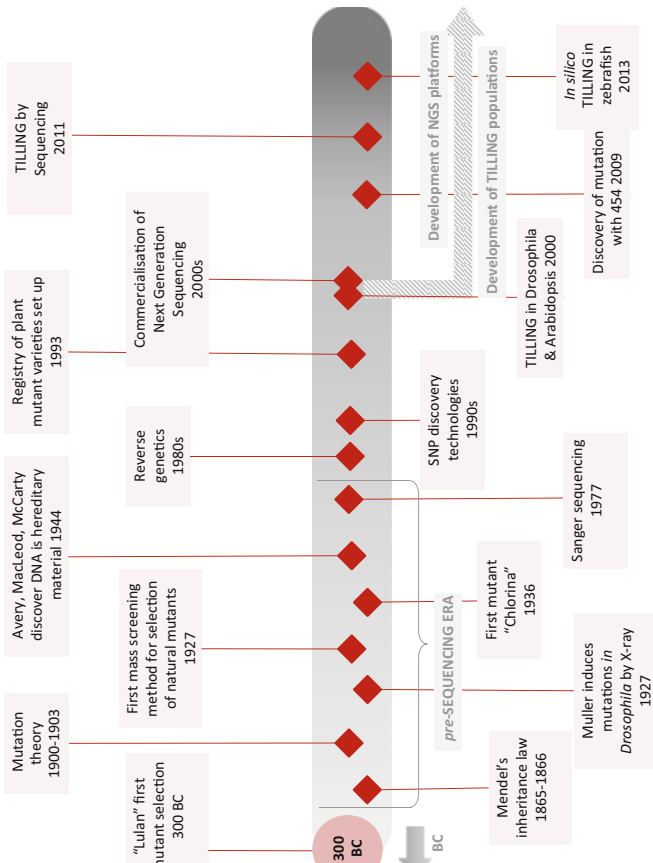


Fig. 1 A time-line of events important for the development of TILLING. The very first documented selection of mutants comes from a Chinese book "Lulan" dated 300 BC [4]. However, the word "mutation" was coined much later by Hugo de Vries. The use of induced mutations to alter plant traits was described by Stadler in the late 1920s. Mutation breeding started in the 1930s with the release of the mutant tobacco cultivar. The recovery of gene sequences began in the 1970s and the first whole plant genome sequence (Arabidopsis) was released in the year 2000. Tens of thousands of genes were annotated with no direct experimental evidence of their in vivo function. Reverse-genetics strategies first developed in the 1980s provided a tool for direct testing of gene function through disruption of specific gene sequences. Single nucleotide polymorphism (SNP) discovery technologies that began in the 1990s allowed the development of reverse-genetics using point mutagens, a method termed TILLING, in Arabidopsis and Drosophila. Following this, there has been an expansion of TILLING into many plant and animal species and the adoption of next generation sequencing to improve mutation discovery throughput. This has enabled the creation of in silico TILLING whereby mutations for every gene in an organism's genome are discovered and catalogued in a searchable database for immediate access by the research community

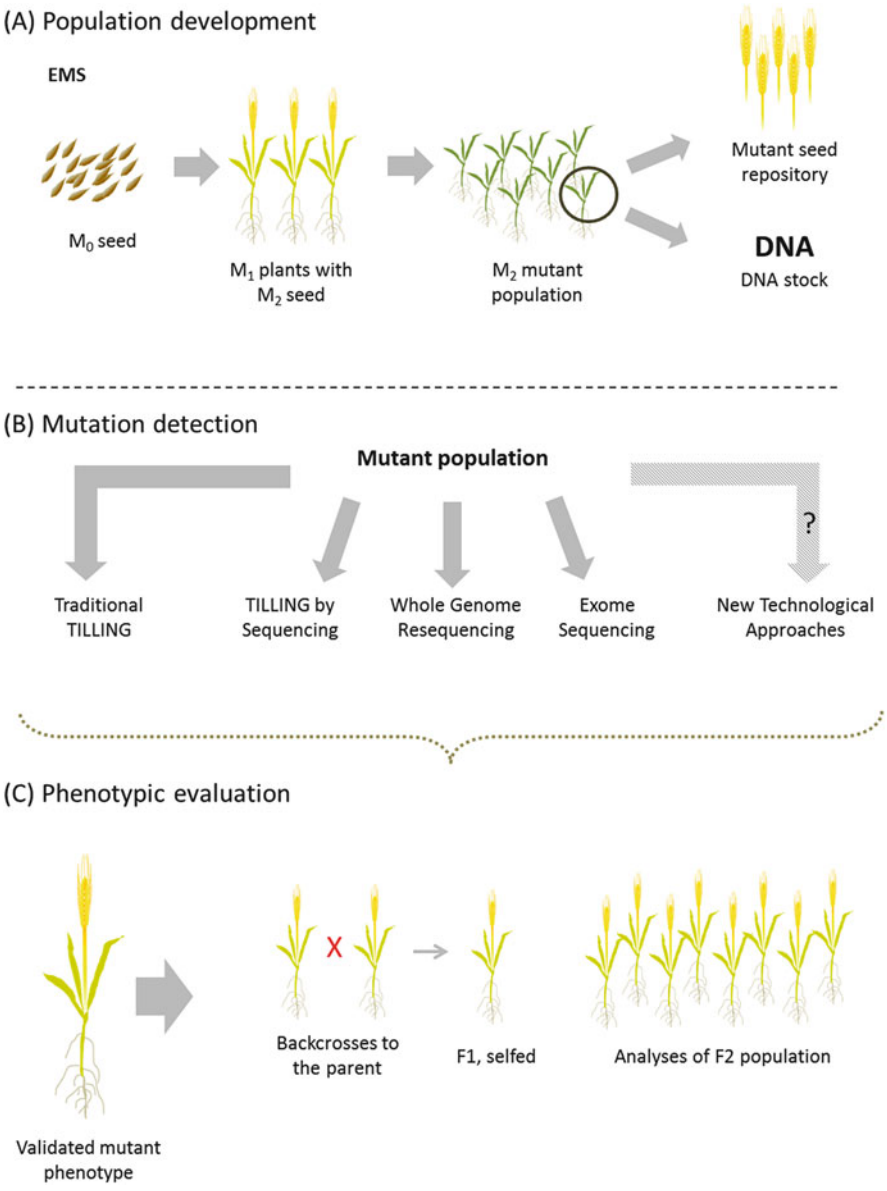


Fig. 2 Overview of the TILLING procedure. The first step is the development of a mutagenized population (**a**). The chemical mutagen ethyl methanesulfonate (EMS) is typically used. The goal is to obtain a high density of induced point mutations while maintaining suitable survivability and fecundity. While seed mutagenesis is common, examples exist of pollen and tissue culture mutagenesis [16, 17]. For seed propagated crops a single-seed descent strategy is often employed so that the maximum mutation diversity can be captured with the minimum of samples to screen. The optimal population size depends on the density and spectra of induced mutations. Higher mutation densities are achieved in polyploids and thus smaller population sizes are required [18–20]. DNA and seed are collected from plants selected for the TILLING population. The time for the

or other sequence element. Some prior knowledge of appropriate targets (genomic sequences) is therefore required. Candidate targets can often be chosen based on homology to sequences in other organisms where some evidence exists of their function. Prior to the advent of TILLING, reverse-genetics approaches had several limitations including the fact that many were species-specific, using, for example, endogenous transposons, or employed transient disruptions that were not heritable [14].

Chemical mutagenesis was first described in the 1940s with the observation of "chemical production of mutations" in Drosophila treated with mustard gas [22]. Mutagens such as EMS became popular and ubiquitous in forward-genetic studies that aimed to elucidate gene function and biological pathways in model organisms. Indeed, many groundbreaking discoveries such as cell cycle control in yeast, segment polarity in Drosophila, and meristematic cell signaling in Arabidopsis were achieved by forward-genetic screens using EMS [23–25]. Chemical mutagens were thus firmly established by the 1980s as compounds that could produce a high frequency of useful, heritable, and stable mutations for gene function studies. Pioneering work using observed phenotypes provided early estimations on the frequency of genic mutations and optimal population sizes when using EMS [26]. Early work also established that EMS induces primarily point mutations in plants [27].

By the 1990s, technologies for rapid and accurate discovery of SNP variations advanced enough to enable the formulation of reverse-genetics approaches utilizing mutagens inducing primarily single base substitutions. The first reports used denaturing high-performance liquid chromatography (HPLC) for the discovery of point mutations in *Arabidopsis thaliana* and *Drosophila melanogaster* [28, 29]. The Arabidopsis group coined the term TILLING (Targeting Induced Local Lesions IN Genomes) for this approach (Fig. 2). This name became widely adopted for subsequent reverse-genetic projects in plants and animals that employed mutagens causing primarily small (SNP and indel) variations [30].

Fig. 2 (continued) development of a TILLING population varies and can take more than 1 year for field propagated crops. The second step of TILLING is screening the DNA library for induced mutations (**b**). Since the inception of TILLING this has been the fastest step. With classical mismatch cleavage and fluorescence detection, an allelic series of ~30-point mutations could be discovered in 1 week using a single DNA analyzer machine [21]. Next-generation sequencing methods have allowed much higher throughputs and the possibility of indexing all mutations in a TILLING population in a short time rather than taking a gene by gene approach. Technologies for DNA sequence evaluation are constantly improving and new approaches will eventually make the discovery and assignment of millions of EMS mutations to individual samples a routine affair. The final step in the TILLING process is testing the effect of the discovered mutations on the mutant plant (**c**). This step is, and will likely remain, the bottleneck for TILLING or any other reverse-genetic approach. Owing to the high density of background mutations induced by chemical mutagens, one or more backcrosses may be needed to unambiguously correlate genotype with phenotype

2 The First TILLING Service and Expansion into Other Plant Species

Immediately upon the first description of TILLING, efforts were made to improve technologies for mutation discovery so that sample throughput could be increased while at the same time reducing false-positive and false-negative error rates. Development and adaption of mutation discovery technologies for TILLING remains an active area of research as described later in this chapter. A major milestone in the early days of TILLING was the adaptation of enzymatic mismatch cleavage (EMC) for SNP discovery. The activity of single-strand-specific nucleases to cleave single-base-pair mismatches had been reported as early as the 1970s [31, 32], but progress and interpretation of the activity of nucleases on single-base mismatches was hindered due limitations in available methods to observe cleaved DNA fragments [33, 34]. Henikoff and colleagues developed a method that paired enzymatic mismatch cleavage, eightfold sample pooling, base-pair resolution denaturing polyacrylamide gel electrophoresis, and laser-based fluorescence detection. This approach was termed "high-throughput TILLING" owing to the fact that 768 mutant plants could be screened for mutations in approximately 1 million base pairs in a single gel run [35]. The method proved to be highly robust and accurate and became widely used for mutation discovery in the first decade of TILLING [30, 36].

The major inputs into a TILLING project are the development of suitably a mutagenized population and the generation of a library of high quality genomic DNAs. It was clear from the initiation of the first TILLING efforts in *Arabidopsis thaliana*, that the TILLING population could become a valuable community resource. The first TILLING service was started in 2001 for Arabidopsis [37]. Users of the service interfaced online with the Arabidopsis TILLING Project (ATP) website. A suite of computational tools guided requestors to choose optimal genic regions of ~1.5 kb to screen for mutations, design PCR primers, and place orders [21]. The ATP would then screen a population of 3,000–6,000 mutagenized lines for mutations in the chosen amplicons, deliver results of alleles discovered, and provide access to seed. In cases where a user requested mutations in a gene that had been previously screened, the requestor was provided a list of mutations already discovered. Thus, within the first year of TILLING being established, one can observe the beginnings of in silico TILLING. The ATP later changed its name to the Seattle TILLING Project as it developed a service for TILLING in *Drosophila melanogaster* and collaborated with other groups to expand TILLING into other species such as rice, maize, and soybean [17, 38, 39]. To date, classical TILLING has been reported for over 25 plant species (Table 1 and [36]). TILLING services expanded as other groups provided screening for a range of different plants including rice, tomato, *Brassica rapa*, *Lotus japonicus*, tetraploid and hexaploid wheat, pea, and zebrafish [55–59]. Facilities have either provided screening for free or have charged a fee to recover costs. One issue with single customer-based cost-recovery services is that they depend on having a minimal number of requests over a set period of time to ensure a stable flow of resources to support staff. Sustainability of

Table 1 Selected examples of TILLING projects

Species (common name[a]), ploidy	Mutagen	Mutation frequency 1/kb	Mutation detection technology[b]	References
Arabidopsis thaliana (Arabidopsis[a]), 2X	EMS	1/200	EMCL	[21, 40]
Arabidopsis thaliana (Arabidopsis[a]), 4X	EMS	1/51.5	Illumina amplicon	[41]
Arachis hypogaea L. (peanut), 4X	EMS	1/967	EMCL	[42]
Arachis hypogaea L. (peanut), 4X	EMS	1/344 kb (single copy) 1/3,028 (multi-copy)	Illumina amplicon	[43]
Brassica napus (canola), 2X	EMS	1/109	Illumina amplicon	[44]
Eragrostis tef (tef), 4X	EMS	1/115; 1/370	454 amplicon	[45]
Helianthus annuus L. (sunflower), 2X	EMS	1/475	EMCL	[46]
Helianthus annuus L. (sunflower), 2X	EMS	1/480	EMCL	[47]
Hordeum vulgare (barley), 2X	EMS	1/1,000	EMCH	[48]
Hordeum vulgare (barley), 2X	EMS	1/500	EMCL	[49]
Hordeum vulgare (barley), 2X	EMS	1/1,333	454 amplicon	[50]
Musa acuminata (banana), 3X	EMS	1/57	EMCL	[16]
Oryza sativa ssp. japonica (rice[a]), 2X	EMS Az-MNU	1/294 1/265	EMCL Illumina amplicon, exome capture/ Illumina	[39, 51, 61]
Oryza sativa ssp. japonica (rice), 2X	MNU	1/135	EMCC	[52]
Triticum aestivum (hexaploid wheat), 6X	EMS	1/24	EMCL	[19]
Triticum aestivum (hexaploid wheat), 6X	EMS	1/38	EMCP Exome capture/ Illumina	[20, 62]
Triticum aestivum (hexaploid wheat), 6X	EMS	1/23.3 to 1/37.5	EMCA	[18]
Triticum aestivum (hexaploid wheat), 6X	EMS	1/34; 1/47	EMCA, EMCP	[53]
Triticum durum (tetraploid wheat), 4X	EMS	1/40	EMCL	[19]
Triticum durum (tetraploid wheat[a]), 4X	EMS	1/51	EMCP, exome capture/Illumina	[20, 62]
Triticum monococcum (diploid wheat), 2X	EMS	1/92	EMCA	[54]
Zea mays (corn[a]), 2X	EMS	1/500	EMCL	[17]

[a]Indicates present or former TILLING service
[b]*EMC (+ symbol)* Enzymatic mismatch cleavage using one type of readout platform, *A* agarose gel, *C* capillary electrophoresis, *H* HPLC, *L* LI-COR, *P* Polyacrylamide gel

public sector TILLING has thus been an issue and several services have already closed down. Development of fully sequenced TILLING libraries as complete in silico resources may be a more sustainable model as it requires only limited labor and resources to maintain databases and seed stocks. This has been possible in recent years through advances in genome sequencing technologies (see below).

One result of the expansion of TILLING into different plant species was the rapid acquisition of data on the effect of chemical mutagens on the plant genome. Keeping in mind that the pre-NGS mutation discovery methods used are highly biased for the recovery of SNP and small indel mutations, data from thousands of discovered EMS mutations showed that for many species the majority of induced changes were G:C to A:T transitions (Table 1, [40]). This supports earlier studies showing EMS alkylating the G residue at the O′6 position resulting in the replication machinery incorporating a T rather than a C in the newly synthesized strand. In some species nearly 100% transition changes have been observed. This deviates in other species, owing possibly to alkylation of other oxygens, variations in DNA repair, and pathways involving depurination [60]. Few mutation hot-spots or regional biases have been reported in studies with data sets large enough to provide statistical significance. Rather, data suggests that EMS results in a generally random distribution of mutations across euchromatic chromosomal locations with some local bias based on adjacent base-pairs [40, 61]. The adoption of next generation sequencing for TILLING screens in the last 5 years has resulted in an increase in datasets on the effect of EMS in plants by two orders of magnitude. The analysis of millions of mutations discovered in wheat will help address the issue of any positional bias in the accumulation of EMS induced changes.

Other chemicals and combinations of chemicals such as sodium azide–MNU have been successfully used for TILLING in plants. Mutation densities reported are similar to that with EMS, while the spectra differ slightly (Table 1). The choice of mutagen may be important in species/genotypes where achieving a high density of mutations with EMS is somehow prohibited due to a cytotoxic barrier or some other effect. Chemical mutagens such as EMS can also result in double strand breaks (DSBs) that could cause larger chromosomal aberrations that were not detected in mutation discovery methods employing PCR amplicons. This is a potentially interesting phenomenon that may be observed when using whole genome or reduced representation genome sequencing approaches. Indeed, analysis suggests that large deletions are induced in polyploid wheat [62]. The frequency of such events is predicted to be quite low compared to SNPs, owing to the fact that large changes will likely be more deleterious, resulting in higher sterility and lower heritability.

3 Next-Generation TILLING

One continual field of study in TILLING has been the development and adaptation of different methods for mutation discovery (Fig. 2). During the first decade of TILLING, numerous publications reported alternative methods for SNP discovery

with the ultimate goal of increasing sensitivities and thus improving throughput and reducing costs. These included capillary and gel-based systems, High Resolution Melt (HRM) analysis, denaturing HPLC coupled with enzymatic mismatch cleavage, conformation-sensitive capillary electrophoresis, and mass spectroscopy [36]. While each method has its advantages and disadvantages, none proved to be such a substantial improvement that it replaced the predominant mode of mutation discovery of enzymatic mismatch cleavage and fluorescence detection. Rather, laboratories adopted the best fit for their purpose based on run-costs, amplicon length, equipment maintenance and automation. This began to change with the commercialization of next generation sequencing. Massively parallel whole genome sequencing coupled with bioinformatics analyses allows rapid discrimination of rare sequence variants versus errors due to the sequencing process [6]. The approach offers a vast improvement on sample screening throughput while dramatically reducing wet bench experiments. Disadvantages include the production of very large data sets, a high bioinformatics load, and higher costs. In addition, much of the cost is spent on sequencing nucleotides outside of genes that will have no phenotypic consequence when mutated.

A natural solution for the discovery of chemically induced mutations using NGS was the adaptation of the original TILLING method of screening PCR amplicons rather than sequencing whole genomes. TILLING remains the same except for the mutation discovery step. Several versions of this have been described (Table 1). All approaches share the goal of maximizing screening throughput by increasing the number of samples screened, the level of pooling, and/or the number of amplicons (total bases of unique sequence) screened. In addition to increasing throughput, sample pooling strategies can also increase the accuracy of mutation calls and allow the determination of the exact individual harboring the identified induced mutation in a pool of samples. Two-dimensional eightfold pooling was used in traditional TILLING screens whereby discovery of a mutation in a row and column pool provided the coordinates of the position of the mutant sample arrayed on a 96-well plate [16]. Higher level pooling is possible with next generation sequencing and so three-dimensional strategies could be considered where samples are arrayed in a cube of stacked plates and mutations are identified in row, column, and plate pools providing the x, y, and z coordinates to identify the exact sample having the mutation. This was used in the TILLING by Sequencing approach described by Comai and colleagues where they screened a total of 768 individual rice mutants in a three-dimensional pool consisting of two dimensions of samples pooled 48-fold and one dimension pooled 64-fold [51]. The group also used TILLING by Sequencing to discover EMS induced mutants in wheat. PCR amplification in this approach closely followed that previously reported for traditional TILLING with single-amplicon reactions performed with pooled genomic DNA [39]. One important issue that was addressed in this work was the fact that higher pooling requires higher amounts of genomic DNA in PCR reactions to ensure that when performing a PCR on a pool, amplification occurs on template DNA from all samples. Failure to achieve this would result in elevated false negative error rates. After PCR products were quantified and then pooled, amplicons were fragmented to an appropriate size for library

preparation, and sequencing was performed using the Illumina platform. Purpose-built bioinformatics tools were also developed for mutation calling and are freely available [63]. Comai and colleagues would also use this method for the development of a tetraploid Arabidopsis TILLING population showing a density of 19.4 mutations per Mb [41]. While many different next generation sequencing technologies have been described, Illumina is currently the most popular for TILLING by Sequencing and exome capture TILLING projects. Haughn and colleagues described a modification of the TILLING approach to identify EMS induced mutations in three-dimensionally pooled DNA samples of polyploid canola [44]. A three-dimensional pooling approach using multiplex semi-nested PCR was described for recovery of sodium azide-induced mutations in rice [64]. Ozias-Akins and colleagues used the TILLING by Sequencing approach, employing two-dimensional pooling, to recover mutations in single and multi-copy stress resistance genes in peanut [43]. PCR products were typically fragmented prior to sequencing because amplicon lengths were greater than available sequencing read lengths.

As read lengths have increased with the Illumina platform it is now possible to consider direct sequencing of amplicons without fragmentation. This may be especially efficient in organisms with small exons such as zebrafish. Moens and colleagues described a strategy for direct sequencing of 250 base-pair amplicons using Paired-End sequencing to find induced mutations in *N*-ethyl-*N*-nitrosourea (ENU) mutagenized zebrafish [65]. Similar work is being carried out using 600 base-pair amplicons and 2 × 300 Paired-End reads to identify EMS induced mutations in tomato [66]. One interesting aspect of the zebrafish work surrounds the type of alleles induced by chemical mutagens. From the start of TILLING, efforts were made to integrate predictions of the effect of point mutations on gene function for optimal primer design and to prioritize identified mutants for phenotypic characterization [67–69]. Owing to the fact that splice-site and nonsense changes are easy to predict, activities surrounded the evaluation of missense changes (where the mutation causes a change from one amino acid to another). In general, only about 5% of EMS induced mutations in an average plant gene will be splice-site or nonsense mutations, and only a fraction of missense changes will be predicted to alter gene function. Therefore, on average more than half of mutations identified in a TILLING screen are expected to be of no value. Why then should efforts be undertaken to identify the individual sample that harbors an unwanted induced mutation? An alternative strategy is to screen larger one-dimensional pools of samples in order to capture all mutations as efficiently as possible. The next step is to evaluate the effect of mutations and choose only those of interest to follow up. This approach was used for zebrafish TILLING. One-dimensional pools of DNA from 288 fish were first screened using the Illumina MiSeq. High throughput genotyping assays (HRM) were then designed for specific genes and all individuals from a pool were screened to identify the one harboring the sought after mutation. A similar approach is being used to identify natural mutations in cassava accessions held at the International Center for Tropical Agriculture (CIAT) and also for TILLING by Sequencing in soybean [70, 71].

4 Towards In Silico TILLING

With advances in next generation sequencing, one can consider developing an in silico resource where all mutations from a mutagenized population are discovered simultaneously and recorded in a database. This is in contrast to traditional TILLING where the user orders mutations in a specific gene prior to screening (Fig. 3).

The in silico TILLING approach allows researchers to get results on available mutations in his or her target gene immediately. The challenge with creating such a resource is that while sequencing costs have reduced, many plant genomes are large and accurate discovery of rare SNP mutations requires a suitable depth of coverage. While examples do exist of whole genome sequencing of thousands of plant accessions in order to uncover natural nucleotide variation, the approach remains cost-prohibitive for most TILLING projects [72, 73]. An alternative way is to sequence only a subset of genomic DNA that is most likely to cause phenotypic variation when mutated (Fig. 4). The first example of this is in zebrafish where DNA was enriched with the annotated exons of all 26,206 protein coding genes [74]. This

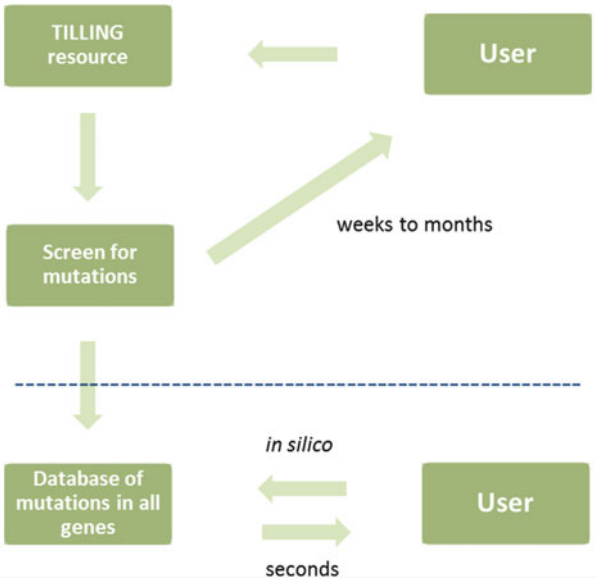


Fig. 3 Traditional TILLING services versus in silico resources. In traditional TILLING services screening for mutations begins when a user requests mutations in a specific gene region (top). Screening of the population is performed for that target and identified mutations are reported back to the user along with information on how to access seed stock. Depending on the speed of the TILLING facility and the number of orders placed, it may take weeks to months before the user receives results [21]. In in silico TILLING, all mutations in a population are discovered and catalogued in a database prior to any user requests. The user searches a database for mutations in the selected gene and results are provided in the time it takes for the search to be completed, typically seconds

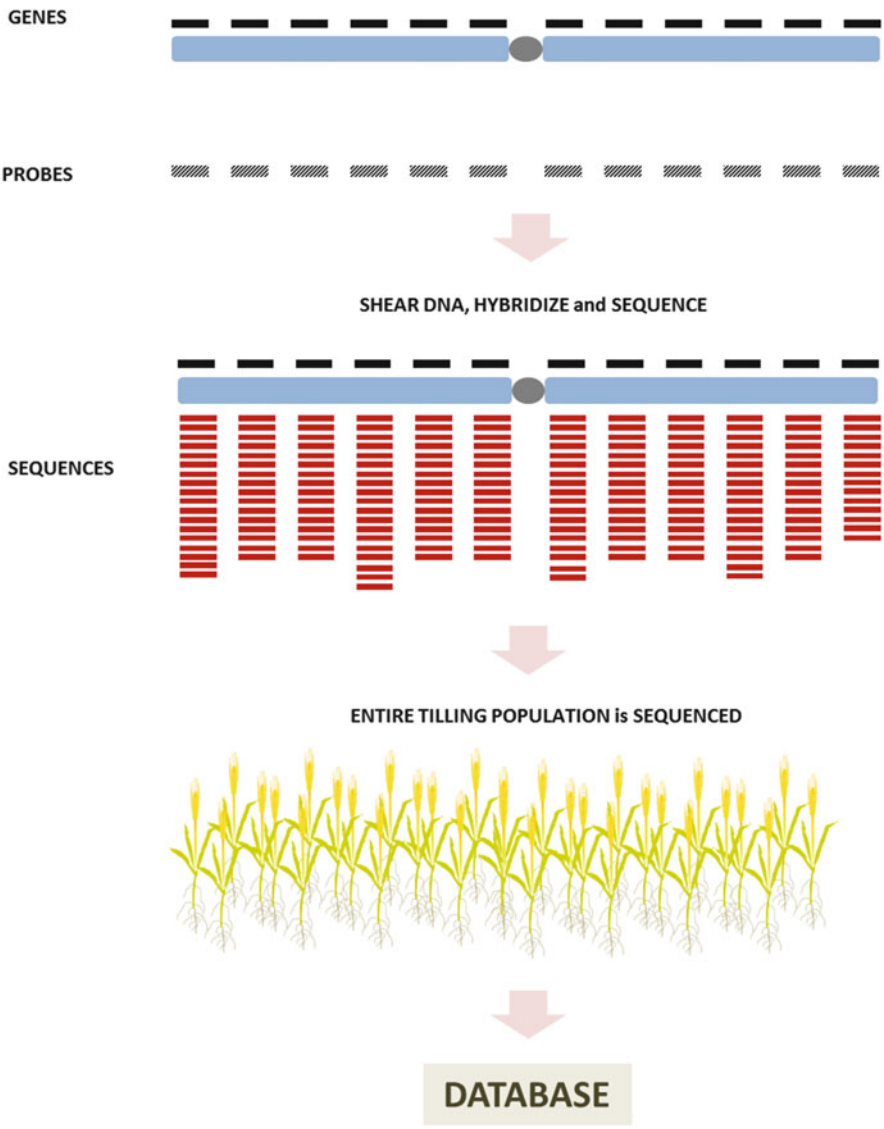


Fig. 4 A simplified example of developing an in silico TILLING resource using reduced representation exome capture sequencing. Probes are designed to cover all genomic sequences of interest (top). Exon sequences that code for proteins are good targets for mutagenesis as the effect of mutations can be predicted in advance. Probes can be designed for any region such as promoters and other regulatory elements. Genomic DNA from mutant plants is isolated, sheared, and then hybridized to the probes. DNA-Probe hybrids are then physically separated and sequenced (middle). This process is performed on the entire TILLING population and a database of mutation information for each plant is created (bottom). Users of the resource access the database and search for mutations in their specific gene target(s). A list of plants harboring identified mutations is returned along with information on how to access seed for the selected plants

covered approximately 60 Mbp of exonic sequence. The size of the zebrafish genome is ~1.4 Gb and so the exome approach represents a major reduction in sequencing loads (about 23×) while not reducing the ability to identify genic mutations.

The approach is especially appealing for large genome plants where there has already been reported success in TILLING. There have been many examples of successful TILLING in polyploid wheat with high mutation densities [18–20, 75]. Reverse-genetics is a powerful approach in polyploids where recessive mutations are not observed due to the presence of homeologous sequences that must also be mutated before a phenotype can be observed. Slade and colleagues combined mutations in starch branching IIa genes in the A, B, and D genomes to produce high amylose wheat [75]. Uauy and colleagues have taken a similar approach of combining mutations in the genomes to increase grain size [76]. An in silico TILLING resource has been produced for both tetraploid and hexaploid wheat. More than 10 million mutations have been reported, making it the largest dataset on the effects of EMS mutagenesis on a plant genome [62]. It is likely that the success of this project will stimulate similar endeavors in other important plants.

5 Reverse-Genetics Using Targeted Genome Editing

Huge progress has been made in targeted genome editing within the past few years [77]. A pubmed search for the term "CRISPR" performed 20 February 2018 showed a total of 8,340 hits with a 30% increase between 2016 and 2017. It is a safe estimation that this number will be much higher and there will be many new breakthroughs by the time this book chapter is published. Targeted genome editing, as the name implies, involves the generation of a genomic change of a precise type in a precise location in the genome of a plant, animal, or microorganism. It is thus a reverse-genetic technique that utilizes induced mutations and therefore shares many similarities to TILLING. With the exception of relatively rare off-target mutations, the approach has the advantage that only the desired change is produced in the organism. A variety of methods and variations on methods have been described including Meganucleases, Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector-based Nucleases (TALENs), and RNA-guided editing using the CRISPR/Cas system [78]. The nucleases create double-strand breaks (DSBs) at desired sequence-specific locations in the genome, following which the DSBs sites are repaired either by non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms that result in the fixation of mutations in the genomic sequence.

Differences between the above-mentioned engineered nucleases have been extensively reviewed [78]. The simplicity of the CRISPR/Cas system has enabled it to become the predominate genome editing method. It is based on the bacterial CRISPR/Cas type II prokaryotic adaptive immune system and uses a Cas9 nuclease and only one engineered single-guide RNA (sgRNA) to specify the target DNA

sequence. In addition to creating novel specific sequence changes, there is an added advantage that homozygous mutations can be immediately produced in a single generation [79, 80]. Further, homeologous loci in polyploid species can be simultaneously edited as was shown by Qiu and colleagues in their work procuring resistance to powdery mildew in hexaploid wheat by mutating three *MLO* loci [81]. Modifications such as CRISPR interference (CRISPRi) and CRISPR activator (CRISPRa) allow modulation of gene expression that can be used for plant studies including pathway analysis of plant stress response [82]. While the focus of this chapter is on plant sciences, it should be noted that the CRISPR based approaches hold tremendous potential to revolutionize human health through the development of disease models and the direct correction of deleterious (disease causing) variants in human cells [83]. Modification of human embryos has been described, something that was merely a trope of science fiction a scant decade ago [84, 85]. The ethical and regulatory issues of using CRISPR approaches in humans, as well as regulatory and social acceptance issues of their use in crops are still being promulgated.

6 Choosing the Best Approach

Given the choices of forward- versus reverse-genetics and random versus targeted mutagenesis, one can consider the comparative advantages of the different approaches to meet breeding and research objectives (Table 2). For example, forward-genetics has been a mainstay of basic research and breeding for decades. Advantages include the fact that it is phenotype driven and no prior knowledge of gene function is required for success. Indeed, the first mutant crop variety was released in the 1930s long before DNA was shown to be the genetic material. There is no intellectual property or regulation when using induced mutations in crop breeding programs and it can be initiated cheaply and easily in any country, including developing ones. This may be one reason why mutation breeding has been so successful and resulted in the addition of billions of dollars to economies [5, 14].

Table 2 A comparison of forward- and reverse-genetics and random versus targeted mutagenesis

	Random mutagenesis and phenotyping	TILLING	CRISPR/Cas
Method type	Forward-genetics	Reverse-genetics	Reverse-genetics
Knowledge of genes/alleles required?	No/No	Yes/No	Yes/Yes
Procedure for inducing variation	Random mutagenesis	Random mutagenesis	Targeted mutagenesis
Target specificity?	No	No	Yes
Regulated?	No	No	No policy yet in some countries
Issues	Possible genetic linkage of induced mutations	Possible genetic linkage of induced mutations	Off target events

Reverse genetics by TILLING requires knowledge of candidate gene targets, but not of specific alleles. An advantage with TILLING is that populations can be prepared in advance where allelic series are available in all genes so that both knockout and missense changes can be recovered by researchers as quickly as seed can be sent from a stock center. Multiple alleles can be tested directly to deepen knowledge on gene function. With advances such as exome capture sequencing, the development of in silico TILLING resources will become inexpensive and common. The major disadvantage with TILLING is the fact that any plant may harbor thousands of point mutations and several backcrosses may be required to unambiguously assign gene function. This is relatively straightforward in genetically tractable crops like cereals but can become extremely challenging in crops like triploid bananas, which are obligate vegetatively propagated. With targeted genome editing approaches one must design and create each mutation. This is considerably more up-front work than random chemical mutagenesis. However, one can avoid the issue of background mutations/linkage drag and make a "clean" variant. Further, the ability to make homozygous lesions has great potential in obligate vegetatively propagated crops like triploid banana, where creating and utilizing recessive alleles is laborious.

When considering forward- versus reverse-genetics and random versus targeted mutagenesis, it is likely that many researchers will not treat these as either/or propositions but rather choose a combinatorial approach that allows the quickest and most cost-effective means to reach his or her goal. One can imagine, for example, using an in silico TILLING resource to first test and validate gene function and then later using CRISPR to create a single mutation in an elite breeding cultivar. This could in some cases be substantially faster than traditional introgression and would avoid any problems with genetically-linked induced mutations that might be present in TILLING lines. The opposite approach could also be taken if targeted genome editing is not desired in the final product. Once genes and alleles are validated by CRISPR, a traditional TILLING population could be created to generate the desired improved trait. Forward-genetics will remain powerful for gene discovery and new sequencing based approaches to cloning mutant alleles will provide information on genes and variants causative for phenotypes that can support reverse approaches [86, 87].

7 Concluding Remarks and Future Perspectives

Genetic mutations and recombination allowed the evolution of species, domestication of plants and animals, and provides the diversity required for modern plant breeding. New technological developments have meant that mutations remain a fundamental tool for both breeder and basic researcher. The advent of reverse-genetics in the 1980s marked the beginning of a new way to use mutations through disruption of specific genes of interest. The vast amount of gene sequence available means that reverse-genetics can be considered for many species. Indeed, whole genome sequences are now available for 47 important crops [88]. TILLING is easily

adapted for most crops as it relies on traditional chemical mutagenesis. A variety of mutation discovery methods can be efficiently used in TILLING screens and so it is expected that TILLING will remain an important approach for functional genomics studies and for breeding. In silico TILLING has been established in wheat, one of the most important food crops. As mutation discovery technologies improve and large-scale sequencing becomes cheap and commonplace, it is expected that in silico TILLING resources will become standard for many plant research communities. Targeted genome editing complements random mutagenesis. As methods such as CRISPR/Cas become routine, the genetic toolkit for many plant species will expand further. This will allow fundamental new biological insights, and also the improvement and domestication of plants that have great potential to help address growing pressures on global food security.

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