Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2798

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **5 (5):** 669-679 (2017)

Review Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Tilling: Is Effective Tool for Mutant Gene Detection in Crop Improvement

Baldeep Singh[1*], Geeta Boken[2] and Geetika Mehta[3]

[1]Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana- 141004

[2]Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar- 125004

[3]Indian Institute of Wheat And Barley Research,Karnal, Haryana 132001

*Corresponding Author E-mail: baldeep.singh55@gmail.com

Received: 5.04.2017 | Revised: 12.05.2017 | Accepted: 23.05.2017

ABSTRACT

Targeting Induced Local Lesions IN Genomes (TILLING) is a functional genomics tool that comprise heteroduplex analysis to detect which organisms in a population carry single nucleotide mutations in specific genes. TILLING is a general reverse genetic strategy that is high in throughput, low in cost, applicable to most organisms and works with a mismatch-specific endonuclease to detect induced or natural DNA polymorphisms in genes of interest. It remains a dominant non-transgenic method for obtaining mutations in known genes and next-generation sequencing (NGS) to the process. NGS will ultimately lead to TILLING. Mutagens like, Ethylmethanesulfonate (EMS), cause stable point mutations and thus produce an allelic series of truncation and missense changes that can deliver a range of phenotypes. Tilling allocates the rapid and cost-effective detection of induced point mutations in populations of physical/chemically mutagenized individuals. EMS is produce G/C to A/T transition by alkylating the G residues and the alkylated G resides to base pair with T instead of pairing with C. In TILLING parental DNA sequence whereas in EcoTILLING genomic DNA sequence is used to identify the mutations created using endonuclease (CEL I). Genes are amplified by PCR using pooled genomic DNA from several individuals as a template. Following denaturation and renaturation of the amplified DNA, heteroduplexes form individuals with wild type and mutant sequence are both present in the pool. The heteroduplexes can be detected by cleavage with different endonuclease and resolution of the resulting fragments on a sequencing gel. TILLING can also be used to detect naturally occurring single SNP's in genes among accessions, varieties, ecotypes or cultivars. The protocols developed for TILLING have been adapted for the discovery of natural nucleotide assortment, a method termed EcoTILLING. EcoTILLING is an extension of TILLING. Current advantages of TILLING have made it an appropriate tool for the detection of both natural and induced variation in several plant species. Here, we review recent headway in this technology for the researchers of plant mutation analysis and genomics era.

Keywords*: Reverse genetics, Mutagenesis, NGS, TILLING, EcoTILLING.*

Abbreviations: ATP = Arabidopsis TILLING Project , bp= base pair, CEL I = Celery nuclease, CMT = Chromomethylase, CODDLE = Codons optimized to detect deleterious lesions, dHPLC = Denaturing high performance liquid chromatography, EcoTILLING = TILLING techniques to look for natural mutations in individuals, EMS = Ethylmethanesulfonate, FAD2 = fatty acid desaturase 2, Hin-a = Hordoindolina-a, HvFor1= *Hordeum vulgare* Floral Organ Regulator, INDELS = Insertions/Deletions), Kbp = Kilo base pair, Mbp = Mega base pair, PTGS = Post transcriptional gene silencing, PCR= Polymerase chain reaction RNAi = RNA interference, SNPs = Single nucleotide polymorphisms, SSCP = Single-strand conformation polymorphism, SSR = simple sequence repeat, T-DNA = Transfer DNA, TILLING = Targeting induced local lesions in genomes.

INTRODUCTION

Chemical mutagenesis has been extensively utilized for the development of cultivars with agronomically important traits in a no. of commercial crop species[4,31]. It has also been employed to obtain mutants for reverse genetic approaches by allowing researchers to examine a range of mutations in a gene of interest including silent, missense, nonsense and deletions and thus establishes an association between a genotype with a particular phenotype[7]. The main constraint in utilizing the chemical mutagenesis as a reverse genetics approach to decipher the function of the sequences arising from the various genome sequencing projects was the lack of an efficient, practical and essentially high throughput method of their detection[3]. TILLING (Targetting Induced Local Lesions in Genome), a high throughput mutation detection method, takes advantage of chemical mutagenesis to generate induced mutations in a plant population, which results in a high mutational density with very low levels of aneuploidy and dominant lethality[15]. TILLING was invented in the late 1990's by a graduate student Claire McCallum while she was attempting to characterize the functions of two chromomethylase gene in Arabidopsis thaliana and since then the method has been streamlined, automated and utilized in many of the important plant taxa[3]. Raising a mutant population through chemical mutagenesis is the first step of TILLING methodology. The seeds are treated with mutagens (generally EMS) and raised to harvest M1 which are self-fertilized to raise the M2 population. DNA extracted from M2 plants leaf tissue is used for mutational screening. The M2 progeny can be self-fertilized and resulting M3 seeds can be preserved in long term storage. The genomic targets are selected based on the web based programmes such as CODDLE which allows user to input the genomic DNA and to evaluate the probable effect of the induced or natural polymorphism on gene function. The primers targeting the genes of interest are thus obtained and used to amplify the target region in a sample of pooled DNA from the mutant population. The forward and reverse primers are differentially labeled with IRD700 and IRD 800 dye. The heteroduplexes formed by the PCR products of pooled samples through denaturation and annealing are detected either through DHPLC, TGGE, ENDO-1, Melt curve analysis technique or CEL-1. CEL-1 is extensively used and it is an endonuclease enzyme isolated from Celery. It not only specifically recognizes mismatches in the heteroduplex but also it cleaves DNA on the 3' side of the mismatch. The cleaved fragments are detected by the presence of the label at one end only and through the size estimation with standard, the location of a mutation can be pinpointed which can be further confirmed through sequencing[3,25,30]. An extension of TILLING is Eco-TILLING which detects alternative mutant alleles arising due to natural mutation and giving rise to DNA polymorphism in species which can't be subjected to chemical mutagenesis[8].

Right from its invention, TILLING has been convincingly proven to be a technique of considerable potential for crop improvement as it represents the use of induced as well as spontaneous mutations in plant breeding. It allows the direct identification of beneficial nucleotide and amino acid changes in genes with known function and their use as the genetic marker for selection[30]. Allelic variation including the null alleles have been generated, detected and characterized in a variety of crops by numerous workers for the improvement of agronomically important traits in Rice[39], Wheat[31,37], Maize[49], barley[5], Rapeseed mustard[38,48], and Soybean[17], Clover[32], Oat[2] and Common bean[29], apart from the model organisms Arabidopsis thaliana and Lotus japonicas.

Cite this article: Singh, B., Boken, G. and Mehta, G., Tilling: Is Effective Tool for Mutant Gene Detection in Crop Improvement, *Int. J. Pure App. Biosci.* **5(5):** 669-679 (2017). doi: http://dx.doi.org/10.18782/2320-7051.2798

The major advantages of TILLING which is used to obtain loss of function or other novel mutations, apart from being very economic in high throughput mutation detection include its non-transgenic approach for the development of cultivars with desired traits. The time and effort needed for the development of mutant population, dose of the mutagen need to be administered to maximize mutation and minimize lethality, false negative and false positive signals, and presence of homoelogous genes in polyploid species are the major disadvantages of TILLING[3].

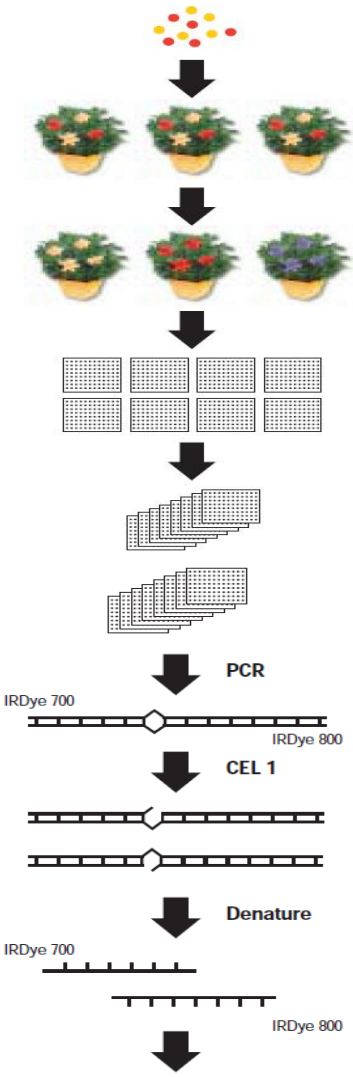
Principle of TILLING-

Targeting Induced Local Lesions IN Genomes (TILLING) is a functional genomics tool that include heteroduplex analysis. Its working on principles which is given below-

1. A mutation is a change of the nucleotide sequence in the genome of an organism which is heritable.
2. TILLING is based on the principle of detection of a mutation based on hybridization of PCR product resulting into heteroduplex formation which indicates the occurrence of mutation at particular site.
3. It is employed to deduce the function of a particular sequence of DNA which has been sequenced using structural genomics technique.
4. Chemical mutagenesis has been extensively utilized in crop improvement programmes.

How TILLING Work

Fig. 1: Typical TILLING Workflow (based on *Arabidopsis* as an model organism



1. Seeds are mutagenized to induce point mutations throughout the genome.
2. A founder population is grown from mutagenized seeds.
3. A founder population is self-fertilized to produce a M2 population.
4. Seeds from the M2 population are stored and DNA samples are collected in 96-well plates.
5. Up to eight 96-well plates are pooled into one and the samples (768) subjected to PCR with two gene-specific primers labeled with different IR Dyes™.
6. Resulting amplicons are denatured and cooled, resulting in heteroduplexes between wild type and mutant samples
7. CEL I nuclease is used to cleave at base mismatches.
8. Samples are denatured and electrophoresis is done. This makes it especially attractive to plant breeders given that the often laborious, relatively low-throughput.
9. In lanes that have a mutation in the pool, a band will be visible below the wild type band on the IRDye™ 700 image. A counterpart band will be visible in the same lane on the IRDye™ 800 image.
10. After detection of a mutation in a pool (lane), the individual DNA samples in the pool are screened again to find out which of the eight pooled samples from the M2 population has the mutation.

(http://www.licor.com/bio/applications/tilling/advantages.html)

Basic Step for TILLING:-

TILLING composed of three main steps: 1) Creation of mutated populations, 2) DNA preparation and pooling, and 3) Detection of mutations in a targeted sequence.

1. Creation of mutated populations:- Plants are ideally suited for TILLING. The ability to store the organism in a dormant state as seed allows for continual mutational screening without the need for continual plant propagation. Additionally, there is a rich heritage of mutagenesis in a variety of plant species, and traditional mutagenesis techniques have been used to create many new crop varieties[21,36].

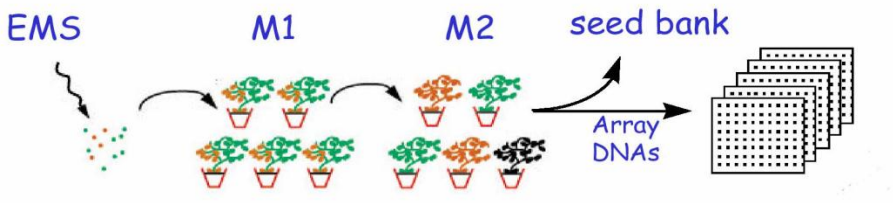


Fig. 2: The basic steps for typical TILLING. Seeds are treated with desired mutagen and then grow M1 population. From M1 population we grow M2 population. DNA is collected from a mutagenized population. DNAs from up to eight individuals are pooled[45]. After extraction and pooling, samples are typically arrayed into a 96-well format[15].

The effect of treatment with EMS is highly expectable, G:C->A:T transition changes represent the majority of induced mutations in most organisms. This is especially striking in Arabidopsis and wheat where > 99% of mutations identified by TILLING are G:C->A:T transitions[15,34]. For many plants, seed mutagenesis is most applicable. Seed are soaked in a dilute solution of chemical mutagen for approximately 10-24 hrs. Due to the multicellular nature of the embryo that is mutagenized, different tissues in the resulting adult plant (termed the "Ml" generation), will hold different genotypes[16]. Mutations in the M1 germline will be heterozygous, and therefore M2 progeny from a selfing of the M1 should segregate mutations in a typical 1:2:1 Mendelian ratio. For Arabidopsis, only one M2 sibling was chosen at random from the progeny of a single M1 self-cross[6].

2. DNA Pooling:-In addition to the density of mutations, sample pooling will directly disturb the efficiency. Factors that affect the ability to pool include the quality of genomic DNA, the accuracy of sample quantification, and the method used for SNP discovery. Various TILLING groups have performed screens utilizing 2, 3, 4, 6, and 8-fold pooling. At the STP, all samples are currently pooled eight-fold. For large scale services, we typically use a one-dimensional pooling strategy where each member sample is signified in only one pool. When a mutation is identified in a pool of eight individuals, each individual of the pool is then screened independently to identify the individual harboring the mutation[6].

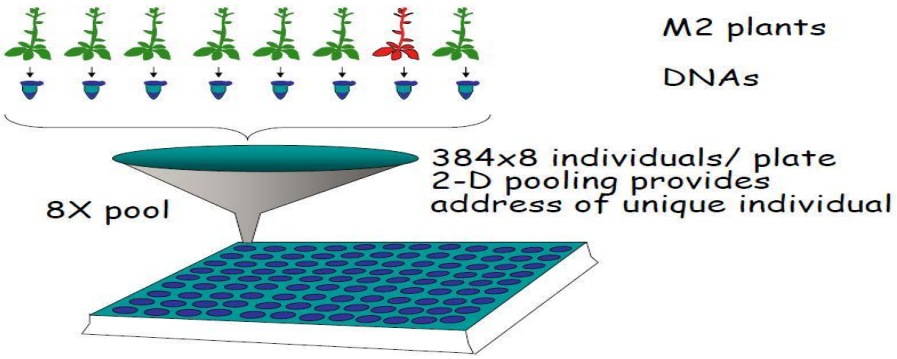
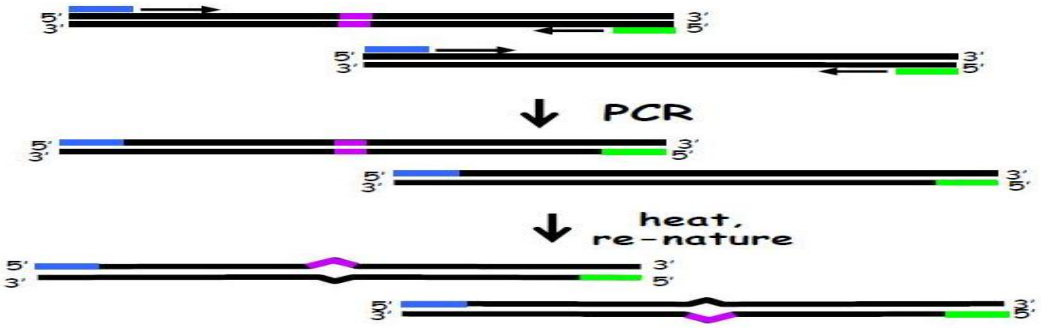


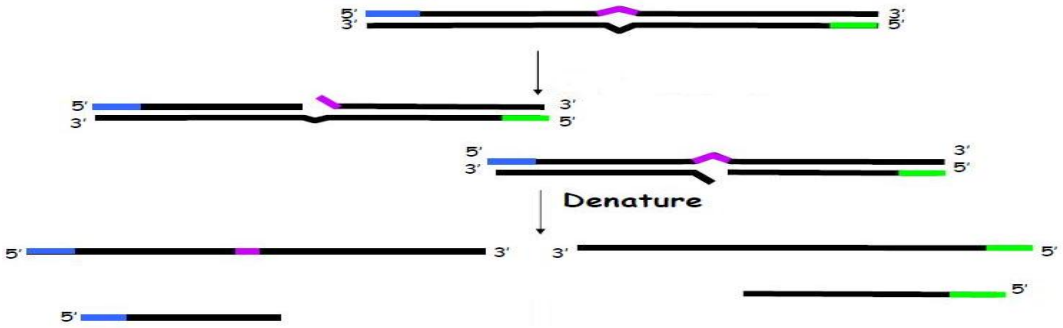
Fig. 3: The other approach is to pool samples two-dimensionally (2-D) such that each sample existing in two unique pools. Also, because two-dimensional pooling involves screening each sample with two-fold coverage, potential false positive and false negative errors are minimized at the initial screening step, rather than when individuals are screened in the second step with one-dimensional (1-D) pooling. To increase throughput the M2 DNA samples are pooled in 8x individuals/plate. Using fluorescently labelled gene specific primers, PCR is carried out on the library of 8x pooled DNA samples. Each primer carries a different fluorescent label. These can then be used to identify amplification products.

3 Detection of mutations in targeted sequence- SNP discovery technologies include array-based methods, denaturing DHPLC, Melt curve analysis, Temperature gradient gel electrophoresis, ENDO-I and CEL-I[7]. Greene *et. al*[15]., screening several thousand mutant individuals will likely be required to ensure a high probability of identifying at least one deleterious mutation (for example see http://tilling.fhcrc.org:9366/files/user_fees.htm l). When a mutation is identified in the pooled DNA, PCR products amplified from the individual DNA samples that make up the pool are sequenced to identify the specific plant carrying the mutation. It consist different steps which are,

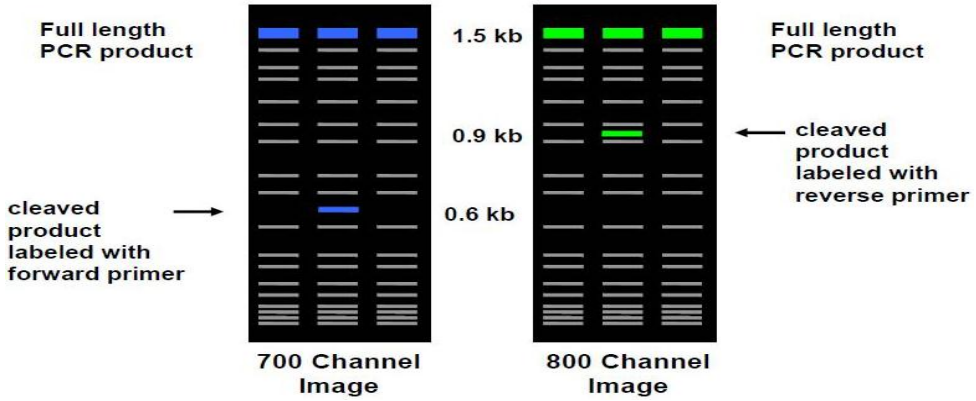
- **PCR amplification of targeted gene-** The target region is amplified by PCR with gene-specific primers that are end-labeled with fluorescent dyes. Figure 3



- **Cleavage with CEL I endonuclease-** Following PCR, samples are denatured and annealed to form heteroduplexes that become the substrate for enzymatic mismatch cleavage. Figure4



- **LI-COR GEL ANALYSER-** Cleaved bands representing mutations or polymorphisms are visualized using denaturing polyacrylamide gel electrophoresis. Cleaved heteroduplexes produced two smaller molecular weight products, one labeled with IRDye 700 and the other with IRDye 800, whose sizes added up to the size of the full length product. Figure5



- **SELECTION OF TILLING TARGET-**

I. Most mutations in non-coding regions like introns, untranslated regions and promoters. it will have no effect on gene function.
II. Probability of obtaining useful mutation can be increased by selecting coding regions of the gene[6,23].
III. Certain software's help in finding appropriate target for mutagenesis for example CODDLE[33,50,51].
IV. In Eco-TILLING, a researcher may choose to maximize intron sequence and poorly conserved coding domains in order to maximize likelihood of finding natural polymorphism[10,22,40,53].

TILLING Applications

For gene discovery:- This technique was first exploited in Arabidopsis TILLING Project (ATP) during 2001. The ATP project has distinguished, sequenced, and delivered over 1000 mutations in more than 100 genes[41]. Through the workshop, mutant materials, DNA samples and mutant information were fully shared by all researchers working on Arabidopsis. Well-developed and tested procedures of TILLING are available for Arabidopsis[25,41], Lotus japonicas[28], maize (*Zea mays* L.)[44], wheat (*Triticum aestivum* L.)[34] and rice[25]. In maize 17 independent induced mutations from 11 genes were obtained from a population of 750 pollen-mutagenized plants[40].

For diversity analysis:- DNA polymorphism extensively exists in various plant species and plays an important role in biological evolution. The methods currently accessible for revealing DNA polymorphism encompass DNA sequencing, single-strand conformation polymorphism (SSCP), hybridization, and microarray. These techniques possess some advantages and limitations. Although DNA sequencing is simple and straight forward, but it is rather costly and time consuming. SSCP delivers a high throughput strategy for polymorphism detection. However, it has low efficiency in detecting novel mutations with a limit of 200 to 300bp length of target DNA sequence. Microarray grips two disadvantages. One is high cost of operation, and the other is the low detecting frequency (< 50%)[5,47].

For functional genomics:- The mutation creates allelic series that can potentially confer a range of phenotype allow structural and functional studies. Conventionally in forward genetics (phenotype to genotype) the gene sequence is finally contingent through selecting large numbers of mutagenized individuals for phenotypic variants. In forward genetic procedures for genome wide analysis primarily for gene coding to a particular phenotype needs a lot of time and work[1]. In reverse genetics (from genotype to phenotype), the gene sequence is known, and mutants are recognized and screened with structural alterations in the gene of interest[27]. In this approach generally less time is required than forward genetics, and its strategies have been effectively used for functional genomics in many plant species.

Modifications of TILLING-

EcoTILLING:- An extension of TILLING is EcoTILLING, Is a method for detecting multiple types of natural polymorphism in natural population. Many crop species cannot be exposed to induced mutation, and EcoTILLING can be used to find the natural variants and their putative gene functions in these crops. This can be done at low cost of SNP/haplotyping methods, and one can screen many samples with a gene of interest[8]. It does not require all the population to be screened to find the polymorphism mutagenize. EcoTILLING in Mla resistance genes of *Hordeum vulgare* (barley) was used in 2006 to examine the allelic variation[26]. It demonstrates how effectively it can be used for the evaluation of diversity in natural populations[12]. It was employed to identify polymorphisms in mung bean.

iTILLING:- iTILLING is a gel free system. Gel electrophoresis is replaced by high resolution melt curve analysis of PCR products to reveal mutations of interest Garvin *et. al*[11].

Deco-TILLING:- Is a modification of TILLING and Eco- TILLING. However, most species have little DNA sequence information and relatively few SNPs have been developed. Which may result in failure to discover SNPs that are useful or efficient for addressing

specific questions. Garvin *et. al*[11]., developed a system to detect SNPs that call DEco-TILLING. The DEco-TILLING method facilitates the development of useful genotyping assays rapidly and inexpensively and can reduce ascertainment bias[41,42,43,46].

PolyTILLING:- TILLING in polyploids Many plants of agronomic importance correspond to polyploid species, including oilseed rape (*B. napus*), cotton (*Gossypium hirsutum*) and both bread and durum wheat (*Triticum aestivum* and *T. turgidum*). In these species, most genes are represented by multiple homoeologous copies, which share a high sequence identity[20].

TILLING: Current status in crops

Conventional mutation breeding, either by radiation or by chemical treatment, has upheld influence on production of many varieties, including high-yielding rice, barley and wheat, etc[13,19]. Besides, it allows not the prompt, parallel selection of numerous genes but also a expecting of the number of alleles that will be documented based on the mutation frequency and library size. The efforts done on plants are reflected as follows.

Arabidopsis thaliana- TILLING was first applied to Arabidopsis thaliana[23,24]. The TILLING Project (ATP) had mentioned 1,890 mutations in 192 target gene. They induce targeted mutations in two chromo-methylase genes that are CMT and CMT2. To facilitate gene modeling and primer design, a computational tool termed CODDLE (Codons Optimized to Deliver Deleterious Lesions, http://www.proweb.org/CODDLE/) was developed. CODDLE finds genomic and protein-coding information from public databases or from the user, constructs gene models, and analyzes them to regulate the region that has the highest density of predicted deleterious nucleotide changes[24]. In a screen for *Arabidopsis* mutants that change methylation and silencing of a densely methylated endogenous reporter gene[6]. *cmt3* mutations confer reduced CNG methylation on these regions. The *cmt3* mutants exhibited enhanced expression and reduced methylation of the reporter gene.

Barley- Caldwell *et. al*[5]., tilled barley which having a fairly large genome size of ~5,300 Mb. It was used for TILLING experiments to find the induced mutations in two genes Hin-an (*Hordoindoline-a*) and HvFor1 (*Hordeum vulgare* Floral Organ Regulator-1) genes were studied, and10 mutants were identified. Among these ten mutants six have missense mutations.

Rapeseed- Two EMS mutant populations of the semi-winter rapeseed were formed for functional genomics in *Brassica napu*, and for introduction of novel allelic variation in rapeseed breeding. Forward genetic selection of mutants from the M2 populations helped in identification of a large number of unique phenotypes. The existing SNPs were used as positive control to discover the distinguishing novel mutations. The method was used to 1344 M2 plants, and 19 mutations were identified[48]. Among them three were functionally conceded with reduced seed erucic acid content.

Zea mays- As part of a NSF-funded research project to ascertain the suitability of plant populations for TILLING, the STP screened maize populations donated by Clifford Weil and Nathan Springer[44]. Samples were screened in four-fold and eight-fold pools using CEL I and the Li-Cor platform. Weil and Monde[49], Identified 319 mutation in 62 genes were having a mutation rate of 0.93/kb. Till *et. al*[40]., used 750 mutagenized plants population to illustrate the function of 11 genes. Six genes having the visible mutation were screened. In this investigation among six genes, a chromomethylase gene DMT102 was studied.

Rice- Rice is an important economic and staple food crop providing about 80% of the caloric intakes of three billion people of the world. Rice genome has ~50, 000 genes[52]. TILLING studies were done to spot the mutations and identify the function of genes, on indica rice using chemical mutagens EMS and MNU. In this study among 10 target genes 57 polymorphism were identified. The use of agarose gel and LI-COR DNA analyzer was finding the induced point mutations[39,52].

Sorghum-The two mutant lines segregated for the predicted brown midrib (bmr) phenotype.

The bmr mutations reduce lignin content of the sorghum stalk, varieties with bmr mutations may also serve as improved bioenergy feedstock for cellulosic ethanol production. The two COMT (caffeic acid O-methyl transferase) mutants (P5A3 and P7H6) are the result of missense mutations within different codons[54].

Soybean- Soybean an important economic crop and a rich source of protein (35-50%) is beneficial for human health[18]. Cooper *et. al*[9]., develop of four independent populations with considerable mutation density, together with an additional method for screening closely related targets, indicates that soybean is a suitable organism for high-throughput mutation discovery even with its extensively duplicated genome. Hashino *et. al.*, generated high oleic acid (>80 %) soybean lines through tilling. A null mutation was created, identified and assembled for the gene gmfad2-1b (which controls the oleic acid biosynthesis) in two mutant lines B12 & E11.

Wheat- Wheat being hexaploid have a large genome size of 12. 000 Mbp is used as an important staple crop, yielding annually 600 million tons. To make a good in quality, partial waxy wheat cultivars are desirable[14]. Slade and colleagues[34] focused on two groups of synthase genes, starch synthase II (Sgp-1) and waxy proteins (Wx). They able to use TILLING to generate a wheat variety with reduced amylose production, which demonstrates the utility of the method for breeding programs. They generate a wheat variety with reduced Amylose content (Useful of High quality of noodles and frozen and backed food). That wheat variety was good for self-life. Slade *et. al*[35]., Developed high amylose content variety through tilling. High amylose lines had reduced expression of SBEIIa[37].

CONCLUSION

TILLING and EcoTILLING are high-throughput and low-cost methods for the spotting of induced mutations and natural polymorphisms. Large-scale TILLING services have distributed thousands of induced mutations to the international research community for individual. Once this individual has been identified, its phenotype can be determined. With increasing pressures on crop productivity predictable in the twenty-first century, we expect the use of induced and natural mutation to elucidate gene function and to enhance phenotypes will continue to advance in importance. Now the successes have been informed in a variety of important plant species, the next task is to use these technologies to develop improved crop varieties. The utility of induced mutations and natural polymorphism has already been recognized for crop breeding, and so the task is mostly one of the implementations.

REFERENCES

1. Alonso, J.M. and Ecker, J.R., Moving forward in reverse: Genetic technologies to enable genome-wide phenomic screens in Arabidopsis. *Nat Rev Genet.*, **7:** 524-536 (2006).
2. Bagge, M., Xia, X. and Lubberstedt, T., Functional markers in wheat. Curr. Opin. *Plant Biol.*, **10:** 211-216 (2007).
3. Barkley, N.A. and Wang, M.L., Application of TILLING and EcoTILLING as Reverse Genetic Approaches to Elucidate the Function of Genes in Plants and Animals. *Current Genomics*, **9:** 212-226 (2008).
4. Boualem, A., Fleurier, S., Troadec, C., Audigier, P., Kumar, A.P., Chatterjee, M., Alsadon, A.A., Sadder, M.T., Wahb-Allah, M.A., Al-Doss, A.A. and Bendahmane, Development of a Cucumis sativus TILLinG platform for forward and reverse genetics. *PLoS One*. **9(5):** e97963. doi: 10.1371/journal.pone.0097963 (2014).
5. Caldwell, D.G., McCallum, N., Shaw, P., Muehlbauer, G.J., Marshall, D.F. and Waugh, R., A structured mutant population for forward and reverse genetics in Barley (*Hordeum vulgare* L.).*Plant J.*, **40:** 143-150 (2004).
6. Colbert, T., Till, B.J., Tompa, R., Reynolds, S., Steine, M.N., Yeung, A.T., McCallum, C.M., Comai, L. and Henikoff, S., High-throughput screening for induced

point mutations. *Plant Physiol.*, **126(2):** 480-4 (2001).

7. Comai, L. and Henikoff, S., TILLING: practical single-nucleotide mutation discovery. *Plant J.*, **45:** 684–694 (2006).
8. Comai, L., Young, K., Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J., Burtner, C. and Oden, A.R., Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. *Plant J.*, **37:** 778–786 (2004).
9. 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. and Henikoff, S., TILLING to detect induced mutations in soybean. BMC *Plant Biol.*, **8:** 9. doi: 10.1186/1471-2229-8-9 (2008).
10. Fuhrmann, M., Oertel, W., Berthold, P. and Hegemann, P., Removal of mismatched bases from synthetic genes by enzymatic mismatch cleavage. *Nucleic Acids Res.*, **30: 33(6):** e58 (2005).
11. Garvin, M.R. and Gharrett, A.J., DEco-TILLING: an inexpensive method for single nucleotide polymorphism discovery that reduces ascertainment bias. *Mol Ecol Notes*, **7:** 735-746 (2007).
12. Gilchrist, E.J., and Haughn, G.W., TILLING without a plough: A new method with applications for reverse genetics. *Curr Opin Plant Biol.*, **8:** 211-215 (2005).
13. Gilchrist, E.J., Haughn, G.W., Ying, C.C., Otto, S.P., Zhuang, J., Cheung D, Hamberger, B., Aboutorabi, F., Kalynyak, T., Johnson, L., Bohlmann, J., Ellis, B.E., Douglas, C.J. and Cronk, Q.C.B., Use of Ecotilling as an efficient SNP discovery tool to survey genetic variation in wild populations of Populus trichocarpa. *Molecular Ecology*, **15:** 1367-1378 (2006).
14. Graybosch, R.A., Waxy wheat: Origin, properties and prospects. *Trends Food Sci Technol*., **9:** 135-142 (1998).
15. Greene, E.A., Codomo, C.A., Taylor, N.E., Henikoff, J.G., Till, B.J., Reynolds, S.H., Enns, L.C., Burtner, C., Johnson, J.E., Odden, A.R., Comai, L. and Henikoff, S. (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. *Genetics* **164(2):** 731-740.
16. Henikoff, S. and Comai, L., Single-nucleotide mutations for plant functional genomics. *Annual Reviews of Plant Biology*, **54:** 375-401 (2003).
17. Hoshino, T., Takagi, Y. and Anai, T., Novel GmFAD2-1b mutant alleles created by reverse genetics induce marked elevation of oleic acid content in soybean seeds in combination with GmFAD2-1a mutant alleles. *Breed. Sci*., **60:** 419–425 (2010).
18. Krishnan, H.B., Engineering soybean for enhanced sulfur amino acid content. *Crop Sci.*, **45:** 454-461 (2005).
19. Kumar, A.P., Boualem, A., Bhattacharya, A., Parikh, S., Desai N, *et al*., SMART—Sunflower Mutant population And Reverse genetic Tool for crop improvement. *BMC Plant Biol.*, **13:** 38 (2013).
20. Lawrence, R.J. and Pikaard, C.S., Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations. *Plant J*., **36:** 114–121 (2003).
21. Maluszynski, M., Nichterlein, K. and Zanten, L., Officially released mutant varieties - the FAO/IAEA Database. *Mut Breed Rev*., **12:** J -84 (2000).
22. Mashal, R.D., Koontz, J. and Sklar, J., Detection of mutations by cleavage of DNA heteroduplexes with bacteriophage resolvases. *Nat Genet.*, **9:** 177–183 (1995).
23. McCallum, C.M., Comai, L., Greene, E.A. *et al.*, Targeted screening for induced mutations. *Nat Biotechnol*., **18:** 455-457 (2000a).
24. McCallum, C.M., Comai, L., Greene, E.A. *et al.*, Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiol.*, **123:** 439-442 (2000b).

25. McCallum, C.M., Comai, L., Greene, E.A. and Henikoff, S., Targeted screening for induced mutations. *Nat Biotechnol*, **18:** 455–457 (2000).
26. Mejlhede, N., Kyjovska, Z., Backes, G., Burhenne, K., Rasmussen, S.K. and Jahoor, A., EcoTILLING for the identification of allelic variation in the powdery mildew resistance genes Mlo and Mla of barley. *Plant Breed.*, **125:** 461–467 (2006).
27. Nagy, A., Perrimon, N., Sandmeyer, S. and Plasterk, R., Tailoring the genome: The power of genetic approaches. *Nat Genet*., **33:** 276-284 (2003).
28. Perry, J.A., Wang, T.L., Welham, T.J. *et al.*, A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume Lotlls japonicllS. *Plant Physiol*., **131:** 866--871 (2003).
29. Porch, T.G., Blair, M.W., Lariguet, P., Galeano, C., Pankhurst, C.E. and Broughton, W.J., Generation of a mutant population for TILLING common bean genotype BAT 93. *J Am Hortic Soc.*, **134:** 348–355 (2009).
30. Rashid, M., He, G.Y., Yang, G.X. and Khurram, Z., Relevance of tilling in plant genomics. *Aust. J. Crop Sci.*, **5:** 411–420 (2011).
31. Sestili, F., Botticella, E., Bedo, Z., Phillips, A. and Lafiandra, D., Production of novel allelic variation for genes involved in starch biosynthesis through mutagenesis. *Mol. Breed.*, **25:** 145–154 (2010).
32. Signor, C.L., Savois, V., Aubert, G., Verdier, J., Nicolas, M., Pagny, G., Moussy, F., Sanchez, M., Baker, D., Clarke, J. and Thompson, R., Optimizing TILLING populations for reverse genetics in Medicago truncatula. *Pl Biotech J*., **7(5):** 430-441 (2009).
33. Slade, A.J. and Knauf, V.C., TILLING moves beyond functional genomics into crop improvement. *Transgenic Research*, **14:** 109–115 (2005).
34. Slade, A.J., Fuerstenberg, S.I., Loeffler, D., Steine, M.N. and Facciotti, D., A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology*, **23:** 75-81 (2005).
35. Slade, A.J., McGuire, C., Loeffler, D., Mullenberg, J., Skinner, W., Fazio, G., Holm, A., Brandt, K.M., Steine, M.N., and Goodstal, J.F.*et al*., Development of high amylose wheat through TILLING. *BMC Plant Biol*., **12:** 69 10.1186/1471-2229-12-69 (2012).
36. Stadler, Mutations in barley induced by X-ray and radium. *Science,* **68:** 186–187 (1928).
37. Stemple, D.L., TILLING: A high-throughput harvest for functional genomics. *Nat. Rev. Genet.*, **5:** 145–151 (2004).
38. Stephanson, P., Baker, D., Girin, T., Perez, A., Amoah, S., King, G.j. and Stergaard, L., A rich TILLING resource for studing gene function in *Brassica rapa*. ,*BMC Plant Biol.*,**10:** 62-68 (2010).
39. Till, B., Cooper, J., Tai, T., Colowit, P., Greene, E., Henikoff, S. and Comai, L., Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biol.*, 7: 19.doi:10.1186/1471-2229-4-12 (2007).
40. Till, B.J., Burtner, C., Comai, L. and Henikoff, S., Mismatch cleavage by single-strand specific nucleases. *Nucleic Acids Res.*, **32(8):** 2632-2641 (2004).
41. Till, B.J., Colbert, T., Tompa, R., Enns, L.C., Codomo, C.A., Johnson, J.E., Reynolds, S.H., Henikoff, J.G., Greene, E.A., Steine, M.N., Comai, L. and Henikoff, S., High-throughput TILLING for functional genomics. *In Grotewold E (ed) Methods in Molecular* Biology, **236:** 205-220 (2003).
42. Till, B.J., Colbert, T., Tompa, R., Enns, L.C., Codomo, C.A., Johnson, J.E., Reynolds, S.H., Henikoff, J.G., Greene, E.A., Steine, M.N., Comai, L. and Henikoff, S., High-throughput TILLING for functional genomics. *Methods Mol. Biol.*, **236:** 205–220 (2003a).
43. 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. and Henikoff, S., Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Res.*, **13:** 524–530 (2003b).

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. and Henikoff, S., Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biol*. **4**: 12 (2004).
45. Till, B.J., Zerr, T., Comai, L. and Henikoff, S., A protocol for TILLING and EcoTILLING in plants and animals. *Nat Protocols*, **1:** 2465-2477 (2006).
46. Till, B.J., Zerr, T., Comai, L. and Henikoff, S., A protocol for TILLING and Ecotilling in plants and animals. *Nat Protoc.* **1:** 2465–2477 (2006c).
47. Triques, K., Sturbois, B., Gallais, S. *et al*., Characterization of Arobidopsis tilaTiano mismatch specific endonucleases: application to mutation discovery by TILLING in pea. *Plant 1* **51:** 1116-1125 (2007).
48. Wang, N., Wang, Y., Tia, F., King, G.j., Zang, C., Long, Y., Shi, L. and Meng, J., A functional genomics resource for *Brassica napus:* development of an EMS mutagenized population and discovery of FAII point mutation by TILLING, *New phytol.*, **180:** 751-765 (2008).
49. Weil, C.F. and Monde, R.A., Getting to the point - Mutations in maize. *Crop Sci*., **47:** S60-S67 (2007).
50. Wienholds, E., and Schulte-Merker, S. *et. al*., Target-selected inactivation of the zebrafish ragl gene. *Science* **297:** 99-102 (2002).
51. Wienholds, E., Eeden, .F, Kosters, M. *et al*., Efficient target-selected mutagenesis in zebrafish. *Genome Res.*, **13:** 2700-2707 (2003).
52. Wu, J.L., Wu, C., Lei, C., Baraoidan, M., Bordeos, A., Madamba, M.R., Ramos-Pamplona, M., Mauleon, R., Portugal, A., Ulat, V.J., Bruskiewich, R., Wang, G., Leach, J., Khush, G. and Leung, H., Chemical- and Irradiation-induced mutants of indica rice IR64 for forward and reverse genetics. *Plant Mol Biol*., **59(1):** 85-97 (2005).
53. Youil, R., Kemper, B. and Cotton, R.G.H., Detection of 81 of 81 known mouse b-globin promoter mutations with T4 endonuclease VII - The EMC method. *Genomics* **32**:431-435 (1995).
54. Zhanguo Xin, Z., Wang, M.L., Barkley, N.A., Burow, G., Franks, C., Pederson, G. and Burke, J., Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biol.*, **8:** 103 (2008).