PHARMATUTOR
PHARMACY INFOPEDIA
ISSN: 2347 - 7881

Review Article

Tilling: Versatile Reverse Genetic Tool

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ABSTRACT

Recent years have been witnessed for exhaustive genome sequencing, nourishing a cause to track the mutation and its consequences at the phenotypic level. But reliability of these genomic studies is the critical issue which is still unaddressed and not properly understood. Moreover *via* TILLING techniques (reverse genetic tool), can be valuable in evaluation of these studies to much extent. Usually TILLING account on a specific gene mutation in order to observe the extent of the functionality of that particular gene at morphological level. This review compiles the literature pertaining to the art of tilling in the evaluation of genomic studies and majorly in concern of functional genomics. Moreover this review also covers the example of the most common species so as to build better understanding of the concept behind this technique of reverse genetics and can be fruitfully applied in extracting the ethano-botanical and therapeutic effect of various medicinal plants.

Keywords: Reverse genetic tool, Tilling, Arabidopsis Thaliana, Agrobacterium T-DNA

INTRODUCTION

Exhaustive techniques exploration decodes the genome sequencing. Most concern belongs to DNA sequencing, BLAST searching and other Bioinformatics tools which retrieve the genetic information and can be inferred in terms of the phenotype level of an organism. But interpretation at phenotypic level is not an easy task and hence it poses a challenging menace to the new discoveries in genomics. To encounter this challenging situation most often implicate technique, RNAi also possesses certain shortcomings e.g. Delivering siRNAs to target loci [1]. In pursuing to overcome this shortcoming, TILLING (Targeting Induced Local Lesions in Genomes) was introduced in the early 21st century where it allow researchers to direct an identification of mutations in a specific gene[2]. Initially it was implicated on a plant model, "*Arabidopsis thaliana*", afterwards a large list of various plants which can be useful for pharmaceutical and phytochemical industry as *via* this technique they directed the gene regulated expression of a particular phytoconstituent. Meantime, this technique can increase the yield of the phytoconstituent or also can increase the adaptability of plant in the stress conditions.

Moreover since it's discovering TILLING has seen considerably transformations and derivatizations which take its application into a new level. Now-a-days it is becoming a conventional technique in a field of reverse genetics which is successfully implicated on corn, wheat, rice, soybean, maize, tomato and lettuce [3]. It works on a very simple principle

How to cite this article: A Negi, Navgeet, BS Gill, SS Anand, Tilling: Versatile Reverse Genetic Tool, PharmaTutor, 2014, 2(1), 26-32

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PHARMACY INFOPEDIA
ISSN: 2347 - 7881

that mutations can be induced via mutagens. Moreover ionizing radiation and certain chemicals can cause genes to mutate and made it possible to perform genetic studies that were not feasible initially when spontaneous mutations were only available! Afterwards the concerning principle was comprehensively implicated for analyzing gene function in order to understand the pheno-geno type relationship of higher organisms. Meanwhile alkylating agents [4] which cause point mutations, valued as their induced mutations brought altered and truncated protein products which can be helpful in precise mapping of gene and protein function.

With the recent expansion of sequence databanks, locus-to-phenotype reverses genetic strategies sufficiently overcome the limitations of phenotypic screens for functional analysis[5]. Even the retrieved sequence alone is sufficient to deduce its function via Bioinformatics comparative tools.

Most common methods for producing a reduction-of-function mutations are antisense RNA suppression and insertional mutagenesis which are currently leading diagnostic tool of functional genomics[3]. As these techniques highly rely on *Agrobacterium* T-DNA vectors for transmission or on an endogenous tagging system, their usefulness as general reverse genetics methods is limited to very few plant species[6]. However, these techniques produce a very limited range of allele types. Meantime, lots of sequencing of Arabidopsis and other organisms urge a call to develop a genome-scale reverse genetic tools which are versatile, automated and capable of creating the wide range of mutant alleles that are needed for functional analysis [2].

Mechanism: In this method, chemically assisted mutagenesis is done to cause point mutation on the targeted loci of a sensitive DNA. TILLING process includes the formation of DNA hetro duplexes followed by amplification with PCR. With amplification, mismatch bubble formation take place between two strands which further cleaved by nucleases. Mismatch plays cardinal role in TIILING and may be induced by different means, see in fig. 1 and fig. 2.

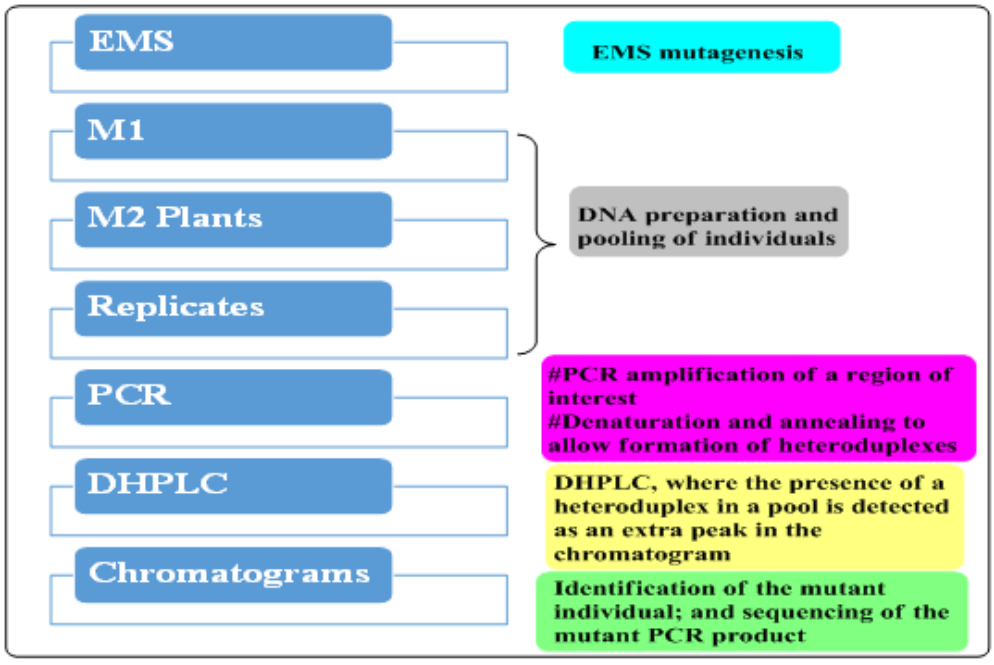

Fig. 1. Concerning steps of mechanism.

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ISSN: 2347 - 7881

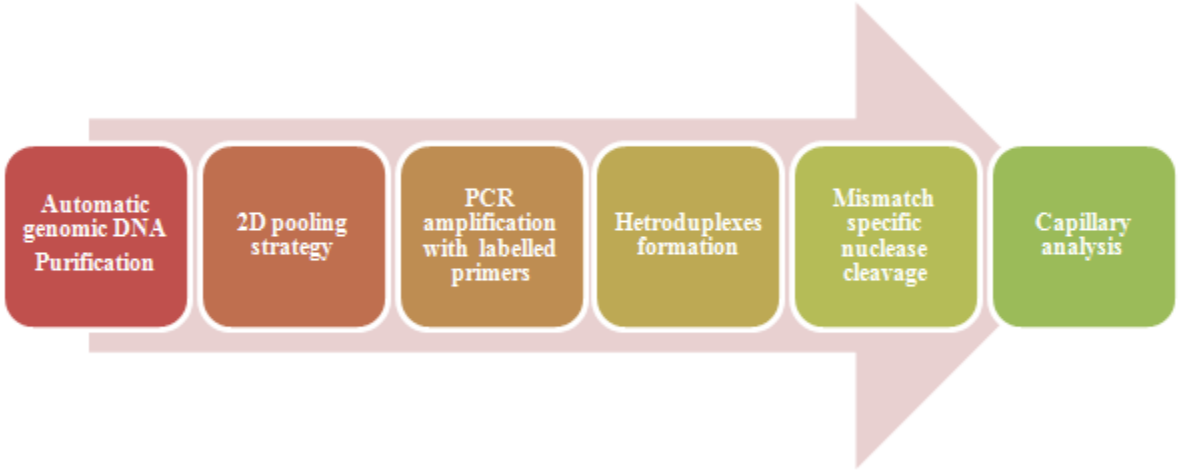

Fig. 2. Different steps involved in TILLING process.

EDGES OF TILLING OVER THE CONVENTIONAL TECHNIQUES

As compared with other conventional transgenic techniques, TILLING always placed aspecific edge in the identification of numerous mutations within the targeted region of the genome where region leads to phenotypes and hence promote the Geno-phenotype studies. In transformation, RNAi although able to provide genetic characterization, but it encountered certain shortcoming during knockout in a series. While TILLING in comparison to RNAi afford consistent analysis by forming a library[7].

ADVANTAGES

- It yields a traditional allelic series of point mutations.
- Very essential for phenotypic analysis of sub-lethal alleles.
- Since it uses chemical mutagenesis virtually all genes can be targeted by screening few individuals.
- Versatile, hence can be applied to virtually any organism.
- It offers high-throughput screening[8].
- Independent of genome size, reproductive system or generation time.
- Assist in detection single base paired polymorphism.

CRITICAL INCIDENCES ARE ALSO AVAILABLE WHERE TILLING WAS MOST FAVORED AND PROVIDE SIGNIFICANT RESULTS

1. Soybean

Chemical mutagenesis was applied to soybean followed by screening for mutations in a target of interest using a strategy known as TILLING. Later on TILLING was employed to 4 mutagenized soybean populations, 3 of which were treated with ethyl methane sulfonate (EMS) and remaining one with N-nitroso-N-methylurea (NMU).

The mutation was discovered in mutagenized soybean populations. Truncation, missense and silent mutations were observed. TILLING was done in following steps:

- Mutagenesis and DNA preparation.
- The primer was designed using CODDLe (Codon Optimized to Deliver Deleterious lesions).
- High throughput TILLING
- LICOR 4200 or 4300 gel images were analyzed using gel buddy [9].

2. Wheat:

Non-denaturing polyacrylamide gel set-up was employed as an alternative for LICOR screens, to make it easy and cost effective[10].

- Generation of EMS mutagenized population.

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EMS = 0.7-0.75%
Germination rates for EMS treated seeds were ~50-60%

● Development of genome specific primers: primers were designed complementary to intron sequences
flanking the target exons and positioned approximately 200bp from sequence of interest.

● Gene libraries were characterized using
*SBEIIa, SBEIIb – starch branching enzyme II genes.
*WKS1, WKS2 – wheat kinase start genes

●Wheat TILLING platform using non-denaturing polyacrylamide detection method.
CODDLe program was used to predict the effect of EMS mutations.
Hexaploid - 40% missense mutations
4.3% truncations
Tetraploid – 28%missense
5.4% truncations

● Comparison between LICOR and non-denaturing polyacrylamide detection methods was done.
LICOR – 1 mutation/40kb
Non-denaturing polacrylamide – 1 mutation / 41.5 kB[11].

3. Tomato: TILLING also provided a platform to increase the production of tomato in term of genetic transformation techniques (GMOs) with desired traits.
Variety: cultivar Red Setter
Doses: ethylemethane sulfonate (0.7% and 1%)
Database: LycoTILL
Mismatch gene: ENDO1 nuclease
The results: Red Setter shows a high - throughput mutation during TILLING.
In tomato, Red Setter was treated with two different doses of ethylmethane sulfonate(0.7% and 1%) and the phenotype data was developed with LycoTILL. This experiment was evaluated by TILLING with point mutation in 7 different genes with mismatch in ENDO1 nuclease. During this experiment, 66 nucleotide substitutions were identified[12].

4. EMS mutagenesis is a straightforward and cost effective way to saturate a genome with mutations. The genus Potyvirus is the largest among plant viruses and consists of widespread and destructive viruses for a number of crops worldwide. The Potyvirus genome has single stranded, positive sense RNA molecule that contains at 5`end a covalently linked virus-encoded protein VPg, replacing the cap structure of mRNA and required for viral infection.
In tomato the role of eIF4E in resistance to 2 Potyviruses, Potato virus Y (PVY), and tobacco etch virus (TEV) was demonstrated by molecular cloning of recessive resistance gene pot-1. Moreover Pot-1 encodes for eIF4E 1 protein and the resistant and susceptible alleles differ by 4 amino acid substitutions [13].

5. Melon
Ripening proteins are conserved across the fleshy fruit species. However the characterization of homologous genes involved in these different species suggested the conservation of similar sort of genetic mechanisms. These genetic mechanisms possess the major share of a Phyto-hormone, ethylene which has a central role in fruit ripening. However ethylene biosynthesis require the conversion of aminocyclopropane-1-carboxylic acid (ACC) to ethylene by the enzyme ACC oxidase regulated by the CmACO1 gene. In case of melon, CmACO1 silencing inhibits fruit ripening and extends fruit shelf life. EMS mutant populations were developed under controlled conditions and established a TILLING platform. Mutations in 11 genes involved in ripening process were screened and CmACO1 that inhibit fruit ripening and extends the fruit storage life.

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BOON to the plant sciences!

Increase the Shelf life of Fruit

Shelf life is a critical factor to preserve the fruits for longer times and preserve its nutritional values. Although majorly Phyto-hormones (especially ethylene) deteriorate the fruits as what's seen in the earlier case of melon [14].However missense genes (L124F and G194D for ethylene biosynthesis, ACC oxidase 1 for fruit maturation has studied well to unwind the mechanism behind the shelf life concept. The outcomes disclosed after these studies revolutionized the molecular biology interface into a new level. These studies showed that the mutation in geneL124F (conservative mutation) didn't affect the gross yield but G194D mutation displayed significant effect as the mutation were at the conserved amino acid. Thus phenotypic analysis showed that the G194D mutant fruit took time\ delayed in ripening with improved shelf life whereas L124F mutation found ineffective.

6. Rice: Rice is the main cereal food source for the majority of the population. Due to increase in population, proportionally demand is also increasing. To fulfill the need, a reliable method is pre-requisitely required to enhance the yield. In this context, molecular geneticist tried exhaustive research in order to attain a target gene which can be later on integrated into the genome at particular loci to increase the productivity without compromising its quality. However TILLING serves the purpose to much extend.

Chemical used: ethyl methane sulphonate (EMS), and the other with a combination of sodium azide plus methyl-nitrosourea (Az-MNU).

Target: 0.7–1.5 kilo bases were PCR amplified using gene specific primers labeled with fluorescent dyes.

Digestion: CEL-I nuclease.

The results: induced mutation density is 2-3 folds higher than previous results[4].

7. Maize:
Mutation segment: 1-kB segments from 11 different genes.

Target gene: DMT102 chromomethylase.
The technique of TILLING was employed on an ethno-botanical valuable species of a crop maize which contain a large genome. Mutations in the DNA samples of 1-KB segments from 11 different genes, obtaining 17 independent induced mutations from a population of 750 pollen-mutagenized maize plants. Genes of interest were the DMT102 chromomethylase gene, in which missense mutation that was predicted to be strongly deleterious[3].

8. Brassica Napus (*B. Napus*; oil seed rape: agronomically important species from the family of model plant *Arabidopsis thaliana. B. Napus*is amphidiploid species containing two diploid genomes originating from a cross between the diploid *Brassica* species, *B. Rapa* and *B. Oleracea.*

Treatment: ethyl methane sulfonate (EMS).

Plant: *Brassica Rapa.*

Genotype: R-o-18 of Brassica Rapa similar to ontogeny to an oilseed rape crop.R-o-18 is self-fertile and produces a large number of seeds per plant as compared to other varieties.

Mutation: Random mutations in genetic material by nucleotide substitution primarily by

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ISSN: 2347 - 7881

alkylation on the O^6 position of guanine leading to GC⟶AT transition changes.

Gene mutated: Six genes with a density of about one per 60 KB.

The results: screening in 1 kB amplicon gives 68 mutations with 97% probability of having a stop - codon mutation[15].

9. *Arabidopsis*:
Analysis: 1900 ethyl methanesulfonate (EMS) - induced mutations in 192 Arabidopsis thaliana target genes.

Detection: twice the number of heterozygotes than homozygotes.

Ratio in results: 3.6:1 indicating selection against homozygous deleterious mutations.

Alkylation: alkylation of G by EMS, >99% of mutations are G/C-to-A/T transitions results from mismatch repairs[16].

10. Soybean :
Soybean (Glycine max L. Merr.) is an important nitrogen-fixing crop which maintains soil fertility and provides protein to world to a greater extent. Less work is done for the improvement of this plant. TILLING is applied in soybean in 4 populations, three with ethyl methanesulfonate (EMS) and one with N-nitroso-N-methylurea (NMU).

Target: 7 in each population (116mutation).

The results: similar mutation in NMU-treated population and one EMS mutagenized population (~1/140 KB), EMS population shows the mutation of ~1/250 kB in their density whereas remaining population observes ~1/550 KB of mutation. This all shows soybean is a suitable plant with high mutation in the genome [9].

11. Caenorhabditis elegans:
Caenorhabditis elegans is a well-established model system in animals. Caenorhabditis elegans is important to study the physiology and biological system which helps the human to combat with different diseases. TILLING was already employed in diagnostic the genetic based phenotype relationship in C. elegans.
For that 1500 individuals were taken and only 10 genes screened for mutations. Whereas the results disclosed 71 mutations(some mutation were silent because of mutations in non-coding DNA or because of wobble amino acid, 59% of the mutations identified are missense allele result in change of only one number of amino acids. Small mutation was observed in some genes i.e. 3% are putative null alleles results in elimination of gene function. Finally it was concluded that forward EMS screens comes to 96% of TILLING mutations were G/C-to-A/T transitions. These results showed a higher rate than forward genetic screens[17].

CONCLUSION

TILLING is powerful reverse genetic technique allows the researcher to find out the critical gene which can afford the improvement in yield and productivity without compromising the quality. It is sensitive, cost effective, high throughput screening technique which is mostly carried out for the study of functional genomics. Success stories of TILLING in different crops indicating its versatility and adaptability and can also be targeted in various studies of medicinal active phytoconstituent of medicinal plants. Further in the future it can rejuvenate the Phyto-chemistry to the new era of Pharmacognosy!

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ISSN: 2347 - 7881

Acknowledgement
Ms. Navgeet acknowledged the Rajeev Gandhi National Fellowship (RGNF) and Balraj Singh Gill acknowledged the Maulana Azad National Fellowship (MANF) for provide the funding during the preparation of this manuscript.

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