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TILLING and Eco-TILLING: A Reverse Genetic Approach to Elucidate Gene Function for Crop Improvement

Karuna*and Navreet Kaur Rai
Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar-125004, Haryana, India

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Abstract

Targeting Induced Local Lesions in Genomes, or TILLING, is a technique that combines chemical mutagenesis, which produces a population with high-density point mutations, with efficient mutational screening in DNApools. It is mostly used as a reverse-genetic approach to determine the function of genes with known DNA sequences andas a non-GMO knockout tool for molecular breeding. The process includes mutagenesis, the development of the TILLING population, DNA isolation and pooling, mutation detection, and phenotypic identification. Numerous choices regarding methodology and modifications can be made in each phase, which will have an impact on the results. Eco-TILLING is a variant of TILLING that examines the allelic diversity of natural populations as opposed to mutant populations. It offers a wide range of potential uses, including genetic diversity studies at specific genes and the search for novel allelic variations and genetic markers in molecular breeding.

Keywords: TILLING, Eco-TILLING, Mutagenesis, DNA pooling

Introduction

Humans have employed nucleotide sequence variation as a key indicator of heritable phenotypic variation for crop development ever since the beginning of domestication. Different populations may create natural variation, or it can be induced by mutagens in life forms where natural variations are limited or cannot be easily recovered, to establish significant genetic divergence. Current agricultural scientists have access to a significant amount of nucleotide sequence data to identify genes of potential agronomic value. The reverse genetic approaches utilize specific gene damage and allow for direct *in vivo* assessment of gene function. Although several reverse genetic approaches have been

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developed, many are restricted in their applicability because they are organism-specific, costly, or only transiently alter gene function.

Modern crop research places a lot of emphasis on utilizing nucleotide sequence diversity for improved crops, aided by the collection of extensive sequence data, further highlighting the requirement for genome-scale reverse genetic approaches for functional analysis. Reverse genetics is a potent approach for identifying gene function and developing new varieties by inducing mutations in specific genes that can then be examined forparticular traits. TILLING (Targeting Induced Local Lesions in Genome) is a non-transgenic reverse genetic approach for identifying induced or natural DNA polymorphisms in regions of interest using a mismatch-specific end nuclease. It combines chemical mutagenesis with PCR-based screening and is applicable to most of plants. TILLING can identify allelic series of miss-sense and truncation mutations using chemical mutagens that produce mostly random point mutations at high density to develop induced populations. Eco-TILLING is a variant that utilizes TILLING methodology to identify natural nucleotide variation associated with critical phenotypic characteristics. Various plant TILLING and Eco-TILLING initiatives are currently under way, demonstrating the relevance of technologies throughout the plant kingdom.

History

In the late 1990s,Claire McCallum, a PhD student at the Howard Hughes Medical Institute and the Fred Hutchinson Cancer Research Centre, began TILLING, to characterize the activity of two chromomethylase genes in *Arabidopsis*. He made an unsuccessful attempt to describe CMT2 using reverse genetic techniques such as T-DNA lines and antisense RNA. The approach that proved to be successful is referred to as TILLING, which was achieved by combining chemically induced mutagenized plants, amplified regions of interest, developing hetero-duplexes among the pooled DNA, and using dHPLC (denaturing high performance liquid chromatography) based on chromatographic changes to identify mutants.

Relevance of TILLING in Genome Analysis

The aim of genome analysis is to comprehend how all the genes of an organism interact and work together. For this reason, knockouts and knockdowns in most, if not all, genes are desired in order to assess the phenotypic effects. Functional inactivation is

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preferred for candidate genes whose function has been determined or is hypothesised to have been determined based on research in model systems or their location in a Quantitative Trait Locus. The hypothesis states that TILLING is an effective reverse genetic method for functional genomics, particularly for crops where other transgenic knockdown knockout approaches cannot be applied.

Requirements for TILLING

1. Establishment of mutagen dose standard.
2. Population TILLING.
3. Gene sequences and candidate gene sequences.
4. High throughput and low-cost system for mutation detection.
5. Functional analysis using Bioinformatics.

Methodologies

TILLING is a high throughput method for identifying SNPs (single nucleotide polymorphisms) and INDELS (insertions/deletions) in a gene or genes of interest in a mutagenized population. As a result, the first step in TILLING is to develop a mutagenized population, which is usually accomplished by using a chemical mutagen. The most effective TILLING mutagen is ethyl methane sulfonate (EMS) which effectively produces single nucleotide substitutions or tiny inserts/deletions (30 nucleotides) in the genome at a high frequency. In the vast majority of TILLING endeavours, EMS is used as the mutagen of choice as EMS therapy is highly predictable in nature. This is particularly apparent in *Arabidopsis* and wheat, where G:C to A: T transitions account for almost 99 per cent of TILLING mutations. The transition mutations (G/C: A/T) are frequent because EMS alkylates G residues, which couple with T instead of the traditional base pairing with C.

Basic Steps for TILLING Process

1. EMS mutagenesis.
2. PCR amplification with fluorescently labelled primers.
3. Denaturation and annealing to facilitate heteroduplex formation.
4. dHPLC, which detects the existence of heteroduplex in a pool.
5. CEL I is used to digest the double-stranded products, which cleaves one of the two strands at the heteroduplex mismatches.
6. Cleaved products are identified on polyacrylamide denaturing gels.

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7. Identification and screening of mutants.

Applications in Crop Improvement

The various applications of TILLING technique in crop improvement are as follows:

- **Genetic engineering:** Agricultural interest in developing phenotypic variations without introducing any foreign gene into the genome of plants. T-DNA and transposon insertions are utilized to create gene knockouts, though they are restricted to only a few crops. TILLING is in front of the transgene which entails finding a large number of mutations in a specific region of the complete genome.
- **Functional genomics:** The identification of numerous mutations in the target region of the genome. Researchers can utilize the TILLING library to search for mutations in their target genes. TILLING is an approach for examining a target gene of interest in any crop without having any knowledge of the gene product.
- **Assessing genetic diversity of natural populations**: TILLING is a technique of introducing valuable genetic diversity into elite germplasm as an alternative to wild relatives. It is also suitable for creating SNPs in a population with a high level of pre-existing polymorphisms.
- **Conserved regions**: Choosing evolutionarily conserved coding regions increases the likelihood of obtaining missense mutations that have negative implications on gene function.
- **Population size**: TILLING is an ideal approach for both small and large populations as it reduces the time and effort necessary to detect mutations.

Approaches used in TILLING

1. Conventional Li-Cor (Gel) based TILLING
   a. DNA pooling
   b. Heteroduplex analysis for mutation detection
2. TILLING by sequencing
   a. DNA super pooling
   b. Construction of Solexa libraries
   c. NGS (Solexa)
   d. Sequence assembly
   e. Mutation detection

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f. De-convolution results of TILLING.

Eco-TILLING

Eco-TILLING (Ecotype Targeting Induced Local Lesions In Genome) is a rapid, inexpensive and well-known allele mining approach.It has been demonstrated to be particularly effective at large-scale mining and high-throughput genotyping of the novel, natural and functional allelic variants of known and candidate genes associated with important agronomic variables in a variety of crop germplasm accessions (without prior knowledge of SNP alleles).

Eco-TILLING is similar to TILLING (Figure1), except instead of findinginduced mutations, it aims to look for natural genetic variation. Eco-TILLING can help in findingnatural variations and their potential gene functions in many species, where chemical mutagenesis is not feasible.

Eco-TILLING can be utilized to determine the level of natural sequence variation across a large number of germplasms, allowing for SNP identification as well as haplotyping. For accelerating agricultural crop genetic improvement, eco-TILLINGs of association and genetic mapping to identify potential novel functional alleles in known and candidate genes/transcription factors (TFs) regulating qualitative and quantitative agronomic parameters has been well established. Eco-TILLING frequently employs a mis-match specific CEL-I nuclease. Most Eco-TILLING methods useleading-edge genotyping technologies to effectively resolve fluorescent dye (IRDye 700/800 and SYBR green)-labeled CEL-I cleaved heteroduplex PCR amplified fragments.

The Eco-TILLING method facilitates marker-assisted breeding for the selection of desirable accessions for crop genetic improvement as well as the identification of potential molecular tags such as alleles and genes/QTLs (quantitative trait loci) controlling key agronomic traits. These methods are incredibly helpful for swiftly and affordably identifying natural polymorphisms or minor variations. It could provide a quick way to reverse genetics by identifying induced and naturally existing variations in a variety of plant species. It has no biosafety issues and is a more cost-effective technology than genetic engineering.

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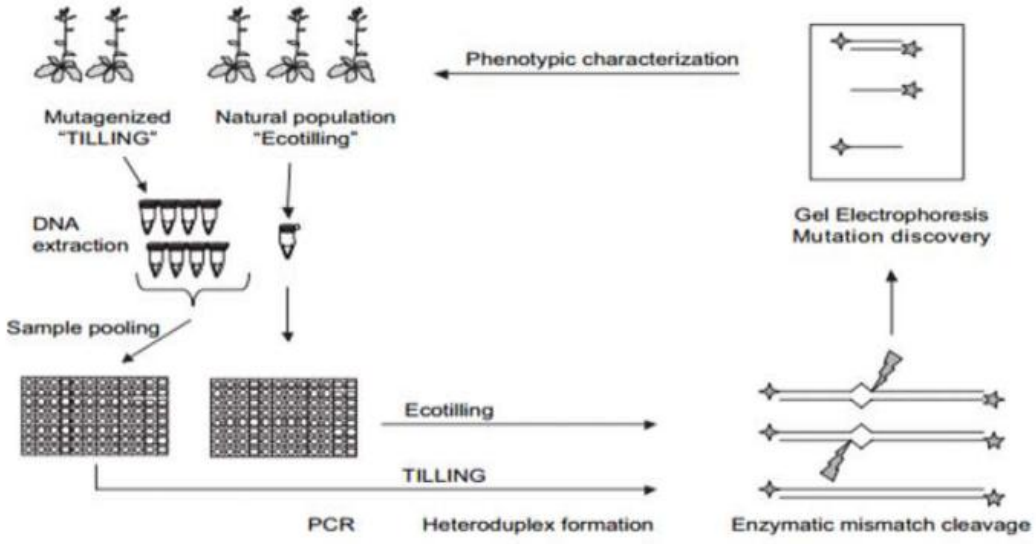

Figure1: General process of TILLING and Eco-TILLING (Kumari *et al*., 2023)

Advantages of Eco-Tilling

It is a useful technique for:

1. Determining heterozygosity.
2. Locating SNPs in germplasm.
3. Identifying disease resistance variations.
4. Detecting natural variations to determine the function of a gene or regulatory element.

Conclusion

TILLING and Eco-TILLING are non-transgenic, high-throughput, and low-cost allele mining approaches for identifying induced mutations and polymorphisms in natural populations. A key benefit of these approaches is thatthe creation of mutation discovery based on standard mutagenesis can be applied to most of the organisms. This method can also be used to enhance the nutritional value and induce disease resistance in crops. The primary limitation of these approaches is the need of sequence information for the construction of gene-specific primers in SNP discovery. TILLING can be used to examine many numerous genes and conserved areas for mis-sense mutations in a small number of studied plant and animal species using sequence data. As a result, the most challenging step will be to use technology to generate and sustain superior crop plants from appropriately mutagenized

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populations. Also, induced mutation and natural polymorphism have already been established as one of the implementation criteria for crop breeding.

Reference

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