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Precision Breeding: Utilizing TILLING and EcoTILLING for Accelerated Crop Improvement

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The urgent need to accelerate crop improvement in the face of climate change, population growth, and nutritional insecurity has redefined breeding strategies worldwide. Among the emerging molecular tools, Targeting Induced Local Lesions in Genomes (TILLING) and its eco-friendly variant EcoTILLING have emerged as versatile, non-transgenic precision breeding approaches that enable the discovery of natural and induced allelic variations. These reverse-genetic tools allow researchers to identify functional mutations in genes associated with yield, stress tolerance, and nutritional quality without regulatory complications linked to GMOs. Recent advances integrating TILLING and EcoTILLING with high-throughput sequencing, genotyping-by-sequencing (GBS), and CRISPR-based screening are revolutionizing mutation discovery pipelines in key crops such as rice, wheat, maize, and barley. This review highlights the principles, applications, comparative advantages, and future prospects of TILLING and EcoTILLING in achieving rapid and precise crop improvement for sustainable agriculture.

Keywords: Precision breeding, TILLING, EcoTILLING, Mutation discovery, Functional genomics, Crop improvement

Introduction

Modern agriculture faces the dual challenge of feeding a growing global population while adapting to climate volatility and depleting natural resources. Conventional breeding, though highly successful, is often limited by long breeding cycles and the random nature of recombination. The advent of precision breeding a suite of tools that allows targeted manipulation and selection of desirable alleles—has transformed crop improvement strategies by enabling breeders to fine-tune traits at the molecular level (Tester and Langridge, 2010). Among the key tools driving this revolution are Targeting Induced Local Lesions in Genomes (TILLING) and EcoTILLING, which represent powerful, non-transgenic reverse genetics approaches capable of detecting induced and natural genetic variation, respectively (McCallum *et al.*, 2000; Till *et al.*, 2003). These approaches have proven particularly valuable in crops with complex genomes or limited genomic resources, offering an accessible alternative to gene editing tools like CRISPR-Cas systems that may face strict biosafety regulations (Comai and Henikoff, 2020).

TILLING was originally developed in Arabidopsis thaliana as a mutagenesis-based reverse genetic tool that combines chemical mutagenesis (commonly using EMS) with PCR-based mutation detection (McCallum et al., 2000). The later development of EcoTILLING extended this concept to the identification of natural nucleotide polymorphisms (SNPs and small INDELs) among diverse germplasm, allowing the exploration of natural allelic diversity without mutagenesis (Comai *et al.*, 2004).

Over the past decade, advancements in next-generation sequencing (NGS), high-throughput genotyping, and bioinformatics pipelines have significantly enhanced the efficiency and scalability of both TILLING and EcoTILLING. Modern "TILLING-by-sequencing" platforms now enable genome-wide mutation discovery at a fraction of the traditional cost and time (Tsai *et al.*, 2022; Yang *et al.*, 2023). These innovations have paved the way for identifying elite alleles conferring drought tolerance, nitrogen-use efficiency, and nutritional enhancement across multiple crop species.

Principles and Mechanisms of TILLING and EcoTILLING

Concept of TILLING: TILLING (Targeting Induced Local Lesions in Genomes) is a reverse genetics approach designed to identify point mutations or small insertions/deletions in genes of known sequence. It integrates chemical mutagenesis with high-throughput mutation screening, enabling breeders to discover allelic variants linked to important agronomic traits without introducing foreign DNA (Till *et al.*, 2003). The technique typically employs Ethyl Methane Sulfonate (EMS), a potent alkylating agent that induces G/C → A/T transitions across the genome, creating a rich source of single-base mutations (Kurowska *et al.*, 2019). These mutations often result in loss-of-function or altered-function alleles, providing valuable material for functional genomics and breeding.

Workflow of TILLING: The TILLING process involves a sequence of well-structured steps:

1. Mutagenesis and Population Development: Seeds are treated with EMS, followed by the generation of M1 and M2 populations. Each M2 plant carries a unique combination of induced mutations.

2. DNA Extraction and Pooling: Genomic DNA is isolated from M2 individuals and combined into pools for efficient screening.

3. Target Gene Amplification: Specific primers are designed for the gene of interest using available genomic data, and PCR is performed to amplify the targeted region.

4. Heteroduplex Formation: The PCR products are denatured and reannealed, forming heteroduplexes when mismatches occur between wild-type and mutant strands.

5. Mismatch Cleavage: Enzymes such as CEL I, ENDO1, or Surveyor nuclease recognize mismatches and cleave the DNA at the mutation site (Triques *et al.*, 2007).

6. Mutation Detection: The cleaved fragments are separated by capillary electrophoresis or visualized through fluorescent labeling and gel imaging. In advanced laboratories, these steps are replaced by TILLING-by-Sequencing (TBS), which employs next-generation sequencing for mutation discovery (Tsai *et al.*, 2022).

Through this systematic approach, researchers can identify mutations in specific genes, rapidly linking genotype to phenotype. Importantly, since the method does not involve transgene insertion, varieties developed through TILLING are considered non-GMO and thus bypass biosafety regulations in most countries (Comai and Henikoff, 2020).

EcoTILLING: Natural Allelic Variation: While TILLING targets induced mutations, EcoTILLING identifies naturally occurring single nucleotide polymorphisms (SNPs) and small indels among individuals of a species (Comai *et al.*, 2004). The workflow is nearly identical to TILLING, except that the population is composed of natural accessions, landraces, or wild relatives instead of mutagenized lines.

EcoTILLING serves several purposes in crop genetics:

- **Diversity Analysis:** Detecting allelic variation across natural populations.
- **Association Mapping:** Linking SNPs to traits such as drought tolerance, yield, or disease resistance (Singh et al., 2021).
- **Conservation Genetics:** Characterizing wild gene pools for rare or beneficial alleles.

An advantage of EcoTILLING lies in its ability to discover functional SNPs directly within elite germplasm without genome modification. Studies in rice, maize, and wheat have identified naturally beneficial alleles associated with nutrient use efficiency and stress tolerance (Yang *et al.*, 2023; Gururani *et al.*, 2024).

Advancements in Detection Platforms: The evolution of detection systems from gel-based to sequencing-based platforms has drastically improved mutation discovery efficiency. Modern TILLING now integrates next-generation sequencing (NGS) and bioinformatics pipelines, such as the Mutation Discovery Portal and Codon Code Aligner, for automated identification of mutations in large populations (Tsai *et al.*, 2022). This transition has reduced cost, improved accuracy, and enhanced the scalability of both TILLING and EcoTILLING across diverse crop species.

Applications of TILLING and EcoTILLING in Crop Improvement

Functional Genomics and Trait Dissection: One of the most significant contributions of TILLING has been in functional genomics helping to connect genes with specific phenotypic traits. Because TILLING allows the discovery of multiple allelic variants for any gene, researchers can study gene function through loss- or gain-of-function mutants. For instance, TILLING was instrumental in elucidating the MLO (Mildew Locus O) gene's role in powdery mildew resistance in barley, paving the way for durable, broad-spectrum resistance (Acevedo-Garcia *et al.*, 2020). Similarly, the FAD2 gene family in soybean, controlling oleic acid content, has been successfully targeted using EMS-TILLING to develop high-oleic acid varieties with improved oil stability (Nguyen et al., 2021).

In cereal crops, TILLING has enabled the discovery of mutants affecting yield and quality traits, such as:

- Waxy starch mutants in maize (Zea mays), improving starch composition for food and industrial use (Hu *et al.*, 2020).
- Low phytic acid mutants in rice (Oryza sativa), enhancing micronutrient bioavailability (Ganie *et al.*, 2022).
- Nutrient efficiency mutants in wheat (Triticum aestivum), increasing nitrogen-use efficiency under low-input conditions (Zhang *et al.*, 2023).

EcoTILLING for Natural Diversity and Association Mapping: EcoTILLING has proven to be a cost-effective approach for exploring natural allelic variation and identifying functional polymorphisms within crop gene pools. It bridges the gap between molecular genetics and breeding programs by rapidly identifying sequence-based markers linked to target traits. In rice, EcoTILLING has been applied to detect SNP variation in drought-responsive genes such as *DREB2A* and *OsNAC6*, aiding in marker-assisted selection for drought-tolerant lines (Ravikiran et al., 2021). In wheat, it has been used to explore polymorphism in genes controlling vernalization (*VRN*) and photoperiod (*PPD*) response, enabling the development of region-specific varieties adapted to diverse agro-climatic zones (Bhatta *et al.*, 2020). Similarly, EcoTILLING in legumes like chickpea and lentil has uncovered allelic variants associated with heat and salinity tolerance, broadening the genetic base for stress resilience (Tadesse *et al.*, 2022). By capturing natural polymorphism, EcoTILLING provides a sustainable way to harness pre-existing allelic variation especially valuable for crops with limited breeding resources or where transgenic modification faces social or legal barriers (Comai and Henikoff, 2020).

Non-Transgenic Breeding and Regulatory Acceptance: Unlike transgenic and gene-edited crops, lines derived through TILLING or EcoTILLING do not contain foreign DNA. Consequently, they are not regulated as GMOs in most countries, simplifying their path to commercialization. For example, TILLING-derived herbicide-tolerant barley and low-gluten wheat have reached advanced breeding stages without biosafety restrictions (Kurowska *et al.*, 2019; Liu *et al.*, 2021). These success stories underscore how mutation-based precision breeding can accelerate trait improvement while maintaining consumer acceptance.

Integration with Modern Genomic Tools: The future of TILLING and EcoTILLING lies in their integration with next-generation sequencing (NGS) and bioinformatics. "TILLING-by-Sequencing (TBS)" platforms now enable whole-genome or targeted region scanning, dramatically enhancing throughput and precision (Tsai *et al.*, 2022). In addition, TILLING and EcoTILLING data can complement CRISPR-Cas and genome-wide association studies (GWAS) by providing natural or induced allelic series for functional validation (Gururani *et* *al.*, 2024). The combined use of these approaches forms the foundation of pan-genomic precision breeding, facilitating targeted improvement across diverse genetic backgrounds.

Conclusion

In an era marked by climate uncertainty and rapid population growth, crop improvement must transcend traditional breeding paradigms. TILLING and EcoTILLING offer breeder-friendly, regulation-light, and highly adaptable tools to uncover and utilize both induced and natural genetic diversity. They empower researchers to translate genomic data into field-ready varieties with improved yield, resilience, and nutritional quality all without genetic modification. The continued integration of mutagenesis, genomics, bioinformatics, and digital breeding will unlock the full potential of these technologies. By bridging classic mutagenesis with modern precision breeding, TILLING and EcoTILLING stand as cornerstones of sustainable crop innovation, ensuring global food and nutritional security for the coming decades.

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