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TILLING and Eco-TILLING in Plant Kingdom

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TILLING (Targeting Induced Local Lesions in Genomes) was began in the late 1990s by Claire McCallum, a Graduate student who used a reverse genetic technique to characterise the activity of two chromomethylase genes in Arabidopsis. TILLING is a non-transgenic reverse genetic, high-throughput method suitable for most plants. TILLING's goals are to find SNPs (single nucleotide polymorphisms) and/or INDELS (insertions/deletions) in a gene(s) of interest in a mutagenized population. TILLING creates mutations by using the same chemical mutagens that have been used successfully in mutant breeding programs for decades. TILLING can find allelic series of missense and nonsense mutations by utilising chemical mutagens that cause mostly random point mutations at high density. PCR and conventional SNP discovery methods are used to target gene areas for mutation identification. The method should be applicable to a wide range of organisms due to the use of general procedures for the creation and discovery of mutations. EcoTILLING is a procedure that uses TILLING methodology to uncover natural nucleotide variation associated to critical phenotypic features. The current article focuses on the techniques of TILLING and EcoTILLING.

TILLING consists of three main steps:

1. **Mutagenization of the Population:** The initial stage in TILLING is to create a mutagenized population, which is usually achieved by using a chemical mutagen like EMS. Many plant species are well suited for this strategy because they can be self-fertilized and seeds can be stored for long periods of time. Seeds are treated with EMS and M_1 plants raised, which are then self-fertilized to create the M_2 generation in plants. Leaf tissues from M2 plants are collected for DNA extraction and then used for mutational screening. To avoid sampling of the same mutation only one M2 individual from each M1 is chosen for DNA extraction. The M2 progeny can be self-fertilized and the M3 produced seed can be preserved in long term storage. For some species, pollen mutagenesis can be measured. This approach was used in generating the populations for the Maize TILLING Project service. The effectiveness and cost of mutation discovery are heavily influenced by the density of generated mutations.
2. **Pooling of DNA:** Sample pooling, in addition to the density of mutations, will have a direct impact on the efficiency and cost of mutation discovery. The two most common pooling strategies have been employed. We commonly utilise a one-dimensional pooling method for large-scale services, where each individual sample is represented in only one pool. When a mutation is discovered in a group of eight individuals, each member of the group is screened separately to find the individual who carries the mutation. Another option is to pool samples two-dimensionally, with each sample appearing in two separate

pools. Potential false positive and false negative mistakes are mitigated *via* two-dimensional pooling, which entails screening each sample with two-fold coverage.

3. **Discovery of Mutation:** Array-based approaches, denaturing HPLC, mass spectroscopy, denaturing gradient capillary electrophoresis, and enzymatic mismatch cleavage are methods of SNP finding technologies. Enzymatic mismatch cleavage and resolution on polyacrylamide gels to detect the cleaved fragments is the most common method used for TILLING. A 1.5-kb gene target is amplified by PCR with gene specific primers in a typical reaction. For downstream viewing, primers are end-labelled with fluorescent dyes. Following PCR, the results are denatured and annealed to form heteroduplexes containing wild-type and mutant DNA strands. Incubation with a nuclease cleaves mismatches, and products are seen using denaturing polyacrylamide gel electrophoresis and a gel readout platform like the Li-Cor DNA analyzer. The most often utilised nuclease is CEL-I, which is derived from celery. CEL-I is a single-strand specific nuclease related to S1 nuclease, and employing standardised TILLING procedures, CEL-I, S1, and mungbean nucleases have all been shown to be suitable for mutation identification.

Once a mutation has been identified, it is sequenced to determine the exact nucleotide change. The mismatch cleavage approach has the benefit of determining the position of each mutation within a few nucleotides. The mismatch cleavage approach pinpoints the region of the putative mutation, allowing for confident detection of any mutation, whether heterozygous or homozygous, with a single sequencing run using the closer of the amplifying primers.

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This approach has been widely applied to the study of functional genomics in plants, particularly in Arabidopsis thaliana, the model plant. TILLING has identified multiple mutations in Arabidopsis thaliana that have offered an allelic series of phenotypes and genotypes to elucidate gene and protein function throughout the Arabidopsis genome.

TILLING has also been used to investigate gene function in Lotus japonicus. TILLING was utilised to look into induced mutations in the SYMRK gene's protein kinase domain, which is required for root symbiosis. There were six missense mutations discovered. TILLING revealed 246 alleles in three waxy gene homoeologues (Wx-A1, Wx-B1, and Wx-D1) from allohexaploid and allotetraploid wheat from allohexaploid and allotetraploid wheat. In rice, EMS and Az-MNU were employed to increase mutational density, with TILLING identifying 57 polymorphisms from 10 target genes.

The TILLING approach has been shown to be effective in identifying variant genotypes and determining gene function in diploid plants with small genomes, such as Arabidopsis. It can also be easily applied to other agricultural plants, such as wheat, that have relatively big genomes that are further complicated by varied ploidy levels.

Eco-TILLING

Eco-TILLING is a molecular approach similar to TILLING, except instead of produced mutations, its goal is to identify natural genetic variation. Because chemical mutagenesis is not feasible in many species, Eco-TILLING can aid in the finding of natural variations and their putative gene functions. This method allows researchers to quickly screen a large number of samples for naturally occurring SNPs and/or tiny INDELS in a gene of interest. The approach has been shown to be effective in detecting DNA polymorphisms, including changes in the number of satellite repeats. Eco-TILLING can also be used to detect heterozygosity levels within a gene fragment in highly heterozygous outcrossing species.

In 2004, work in *Arabidopsis thaliana* resulted in the first publication of the Eco-TILLING method, which adapted TILLING to mine for natural polymorphisms. Eco-TILLING was also used to find polymorphisms in a mung bean germplasm collection.

Conclusion

Both TILLING and EcoTILLING are high-throughput and cost effective techniques for the discovery of induced mutations and natural polymorphisms. The methods are general and have successfully been applied to many plants, including crops, as well as micro-organisms and animals also. After achieving success in a number of key plant species, the next step will be to apply this technology to the development of enhanced agricultural varieties. Because the efficacy of induced mutations and natural polymorphism for crop breeding has previously been proved, the task is largely one of implementation.

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