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High throughput screening for induced point mutations byTILLING in Plants

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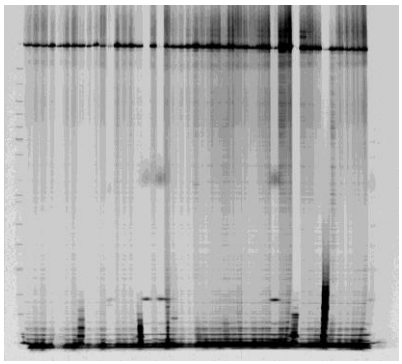

TILLING LICOR GEL IMAGE

ABSTRACT
TILLING, which is a reverse genetic high throughput approach, aims to identify SNPs (single nucleotide polymorphisms) and / or INDELS (insertions / deletions) in a gene / genes of interest from a mutagenized population. Therefore, the first step in TILLING is the creation of a mutagenized population, which is often accomplished by treatment with a chemical mutagen such as 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; however, multiple strategies also exist for the creation of mutant populations in animal species. In plants, seeds are treated with EMS and grown out to produce M1 plants, which are subsequently self-fertilized to produce the M2 generation. 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 resulting M3 seed can be preserved in long term storage. EMS typically produces transition mutations (G/C : A/T) because it alkylates G residues [Comai, 2006] and the alkylated G residue pairs with T instead of the conventional base pairing with C [Henikoff, 2003]. It is a beneficial strategy for users to try a range of concentrations of the chemical mutagen being applied to evaluate the toxicity and sterility on germinal tissue before preparing large mutant populations.

Keywords: TILLING, *SNPs, INDELS, EMs.*

1. INTRODUCTION

The gene function the expanding portfolio of genomic technologies was applied for post-genomic sequencing era. Sequence analysis identified by targeted inactivation of genes by reverse genetic approaches. Most common tool for traditional genetic analysis by chemical

mutagenesis and screening is probably reverse genetics which include TILLING (for Targeting Local Lesions IN Genomes). The genomes of mutagenized organisms for mutations in a gene, typically single base-pair substitutions searches by TILLING. Several publications and reviews (e.g. Gilchrist and Haughn, 2005; Stemple, 2004) of TILLING emerged as a robust approach for the application of general strategy in Arabidopsis thaliana (McCallum et al., 2000) and Drosophila melanogaster (Bentley et al., 2000). Briefly, TILLING provides a range of mutant alleles and is potentially applicable to any organism that can be effectively mutagenized. TILLING can be applied to species for which genomic resources are limited. This review is to inform practical aspects of the technology for developing the mutagenized population to mutation discovery for the research use. Mutation discovery for the TILLING strategy for the improvement to current protocols and the impact of new genomic screening methods.

Induced mutations using by the geneticists from past 75 years ([Muller 1930, Stadler 1932). Genetic mutation is a powerful tool that establishes a direct link between the biochemical function of a gene product and its role *in vivo*. By mutagenesis, identification of genes and the function of their products can be determined by isolating and studying mutants that are defective in specific process pathway. Among the mutagens that have been used to induce mutations, chemical agents play a major part and have become especially popular in use. Ethyl methanesulfonate (EMS), an alkylating agent, is particularly effective because it forms adducts with nucleotides causing them to impairs with complementary bases, thus introducing base changes after replication [Ashburner, 1990]. Furthermore, the effect of EMS mutagenesis often results in a large number of recessive mutations across the genome. Other alkylating agents such, as ethylnitrosourea (ENU), have also been used to effectively induce non-specific mutations [Lightner, 1998]. A specific advantage of EMS mutagenesis is that a series of allelic mutations can be obtained, displaying a range of phenotypes that can serve as the basis of detailed structure functionstudies.

In *Arabidopsis*, five percent of EMS-induced mutations in targeted coding regions result sudden termination of the gene product, whereas fifty percent result in missense mutations that alter the amino-acid sequence of the encoded protein [Greene, 2003]. The mutations by EMS mutagenesis are generated randomly genome-wide and can allow for a high degree of mutational saturation without the excessive collateral DNA damage that may cause aneuploidy, reduced fertility, and dominant lethality. Given these advantages, chemical mutagenesis has maintained its popularity, even with the advent of sophisticated transgenic techniques. Briefly, providing a range of mutant alleles makes TILLING method potentially applicable to any organism that can be mutagenized. Also, species which transgenic methods are limited or not applicable can be used in TILLING applications.

OVERVIEW OF THE TILLING METHOD

TILLING first began in the late 1990's from the effort of a graduate student, Claire McCallum (and collaborators from Fred Hutchinson Cancer Research Center and Howard Hughes Medical Institute), who worked on characterizing the function of two chromomethylase genes in *Arabidopsis*. Claire McCallum utilized reverse genetic approaches such as T-DNA lines and antisense RNA, but was unable to successfully apply these approaches to characterize CMT2. The approach that was successfully turned out to be what was known as TILLING (Targeting Induced Local Lesions in Genomes). This was accomplished by pooling chemically induced mutagenized plants together, amplifying the region of interest, creating heteroduplexes among the pooled DNA, and performing dHPLC (denaturing high performance liquid chromatography) to detect the mutants by chromatographic alterations. Since the inception of this method, TILLING has been streamlined, automated, and utilized in many plant and animal taxa.

TILLING, which was a reverse genetic high throughput approach, aims to identify SNPs (single nucleotide polymorphisms) and / or INDELS (insertions / deletions) in a gene / genes of interest from a mutagenized population. The first step in TILLING is the creation of a mutagenized population, which is often accomplished by treatment with a chemical mutagen such as EMS. Many plant species are well suited for this strategy because they can be selffertilized and seeds can be stored for long periods of time, multiple strategies also exist for the creation of mutant populations in animal species. In plants, seeds are treated with EMS and grown out to produce M1 plants, which are subsequently self-fertilized to produce the M2 generation. 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 resulting M3 seed can be preserved in long term storage. EMS has been widely used as a chemical mutagen in TILLING in both plant and animal studies to generate mutant populations, although other chemical mutagens can be effective . EMS typically produces transition mutations (G/C : A/T) because it alkylates G residues and the alkylated G residue pairs with T instead of the conventional base pairing with C. It is a beneficial strategy for users to try a range of concentrations of the chemical mutagen being applied to evaluate the toxicity and sterility on germinal tissue before preparing large mutant populations.

Once the population has been prepared, the genomic DNA targets need to be selected. The web based program CODDLE (http://www.proweb.org/input) allows users to input genomic, cDNA, or protein sequences and evaluates the probable effect of induced or natural polymorphisms on gene function. Optimal PCR primers can also be designed for a functional domain target. The next step was to collect DNA from the population and normalize the DNA concentration. It was crucial to ensure that all DNA samples are equivalent so that no biasing of samples occurs. Once samples have been normalized, they can be pooled together. In general for diploid organisms, a pool of DNA can contain up to eight individual samples in the pool and be successful in mutation detection. With a larger pool size, the sensitivity of mutation detection will decrease, because the proportion of heteroduplexes compared to homoduplexes in the reaction was reduced. Therefore, depending on ploidy level, heterozygosity, and the amount of naturally occurring SNPs, optimal pooling for a species of interest should be determined empirically.

Once the pooled DNA was arrayed into 96 well microtiter plates, pooled samples are amplified using primers targeting the gene of interest. The forward and reverse primers are differentially 5' end labeled with IRD700 and IRD800 dye labels for fluorescent detection at ~700 nm and ~800 nm, respectively. Next, heteroduplexes and homoduplexes are formed from the PCR products of pooled samples (consisting of mutants and the wild type) by heating (denaturing) and cooling (annealing). The endonuclease enzyme CEL I is applied and a short incubation is required for the enzymatic reaction to progress. CEL I, isolated from celery, not only specifically recognizes mismatches in the heteroduplex, but it also cleaves DNA on the 3' side of the mismatch. After the enzyme incubation period, detection of any digested fragments occurs by separation on a denaturing polyacrylamide gel attached to a LI-COR 4300 DNA analysis system. Pools containing an induced mutation will consist of a mixture of homo- and heteroduplexes. Therefore, when fragments are separated a full length product (detected in both 700 and 800 channels) and two cleaved fragments (one IRD700 labeled, one IRD800 labeled) will be visible. The sum of the cleaved fragments should equal the full length PCR product. The size of the cleaved fragments can be estimated by comparison to a size standard, and thus, the approximate location of the mutation will be identified and further confirmed by sequencing. The web based programPARSESNPhttp://www.proweb.org/parses np/ can be used once mutations are identified to

display the locations of the polymorphisms in a gene/ genes in a graphical format.

TILLING IN PLANTS

TILLING method has been widely used for the study of functional genomics in plants, especially for the model plant *Arabidopsis thaliana*. In 2003, Greene *et al*. reported that the *Arabidopsis* TILLING Project (ATP), which was set up and introduced as a public service for the *Arabidopsis* community, had detected 1890 mutations in 192 target gene fragments. Heterozygote mutations were detected at twice the rate of homozygote mutations. Therefore, the mutational density for treatment of *Arabidopsis* with EMS was approximately 1 mutation / 300 kb of DNA screened with these mutations distributed throughout the genome. The numerous mutations in *Arabidopsis thaliana* that have been identified *via* TILLING have provided an allelic series of phenotypes and genotypes to elucidate gene and protein function throughout the genome for *Arabidopsis* researchers. Another model plant, *Lotus japonicus*, has also been the focus of elucidating gene function through TILLING. *Lotus japonicus* is a perennial temperate legume that is a model plant for genomic studies because it has a short life cycle, is a diploid ($2n = 2x = 12$), has a relatively small genome (472 Mb), and was self fertilized. TILLING was used to investigate induced mutations occurring in the protein kinase domain of the SYMRK gene, which is necessary for root symbiosis. Six missense mutations were identified along with a mutation in the splice acceptor site. Nitrogen fixation and the functional role of sucrose synthase was the target of another *Lotus japonicus* TILLING study. Six isoforms of sucrose synthase were identified and several mutations including missense and nonsense were located in four of the six isoforms. Quantitative RT-PCR was performed to examine expression levels in *L. japonicus*, which were determined to have differential expression in various plant organs. Furthermore, EMS null allele mutants were examined and shown to have reduced sucrose synthase activity compared to the wild type; however, mutants still retained the ability for nitrogen fixation. In a separate study of pea (*Pisum sativum*), which also fixes nitrogen and is a member of the legume family, TILLING was applied to identify an allelic series of mutations in five genes with a total of 60 mutants identified. Some of the mutations discovered in the LE gene, which encodes 3β-hydroxylase, were further characterized and determined to affect internode length. Mutants were backcrossed to the wild type and the segregation of the mutations and their respective phenotypes were examined.

TILLING has radiated from the model plants such as *Arabidopsis* with a simple small diploid genome (125 Mb) to other agronomically important crop plants with more complex genomes due to the success of ATP (now known as Seattle TILLING Project, STP). In 2004, maize, which was an important staple crop with a large genome, was shown to be conducive to the TILLING method. A total of 11 genes were examined in a population of 750 mutagenized plants and six of these 11 genes had detectable induced mutations. One of the genes examined in this study, DMT102 (chromomethylase gene), has been previously suggested to play a role in non-CpG DNA methylation and gene silencing in *Arabidopsis* [46]. A Maize TILLING Project established in 2005 at Purdue University has already identified 319 mutations in 62 genes, which has greatly assisted functional genomic studies in maize. Barley, which is also an important cereal crop with a fairly large genome size of ~5,300 Mb, was evaluated for the ability of induced mutations to be detected by TILLING. Two genes (*Hin-a* and *HvFor1*) were examined and 10 variants were identified, six of which were missense mutations. Phenotyping the M3 individuals demonstrated that 20% had visible phenotypes. Wheat is an extremely important agronomic staple crop with an estimated production level of 600 million tons per year. A polyploid plant investigation to locate variants in the *waxy* locus (granule-bound starch synthase I, GBSSI) in wheat was implemented. Partial *waxy* wheat cultivars are desirable because production of amylose starch was reduced, which leads to the production of superior flour and noodle products for human consumption. Wheat genetics can be complicated because its genome is complex, it is an

allohexaploid, and the total genome size is quite large (17,000 Mb). A total of 246 alleles were uncovered in three *waxy* gene homoeologues (*Wx*-A1, *Wx*-B1, and *Wx*-D1) from allohexaploid and allotetraploid wheat *via* TILLING. This comprehensive allelic series provided 84 missense, three nonsense and five splice site mutations. Phenotyping of M3 progeny demonstrated reduction of amylose production. Detecting genetic variants *via* phenotyping in wheat can be difficult because redundant copies of loci in the genome can mask expression. This study identified more extensive allelic variation in GBSSI than was identified in any report produced in the last 25 years.

Rice, which is also a staple and important economic crop around the world, currently estimated to provide 80% of the caloric intake for three billion people, has been the focus of a few TILLING studies. The rice genome has been predicted to contain ~50,000 genes, of which gene function needs to be determined empirically. In 2005, a report was published on the generation of a large mutation population (60,000) using multiple chemical mutagens on IR64, a widely grown *indica* rice. This study demonstrated that TILLING was suitable for reverse genetic studies with mutations detected in two genes; albeit, the mutational density in the population was fairly low. In addition, extensive phenotypic variation was assessed for the various chemical mutagens used to develop the mutant population and albinism was a common phenotype no matter which mutagen was applied. In the study, EMS and Az-MNU were used to induce an elevated mutational density in rice, with 57 polymorphisms identified from 10 target genes by TILLING. Another report on rice TILLING published in 2007, demonstrated the efficacy of TILLING to detect mutations by separation of products on agarose gels. Results were analogous to pooling DNA and detecting mutations on a LI-COR DNA Analyzer.

Soybean (*Glycine max*) contains approximately 35-50% protein and has been shown to be beneficial for human health. It was a important economic crop that can improve soil quality by fixing nitrogen. Four mutant populations from two genetic backgrounds (Forrest and Williams) were created for soybean by treatment with EMS or NMU and evaluated for induced mutations. Several of the target genes initially tested amplified more than one target. Further work was carried out to produce a single product to employ TILLING so that mutation detection functioned optimally. A total of 116 mutations were identified *via* TILLING from seven target genes. The majority of the mutations uncovered by TILLING were determined to be the expected G/C to A/T transitions.

Even though TILLING was originally designed for and applied mainly in *Arabidopsis* in the early years of its inception, it has been demonstrated to be an extremely versatile approach compared to many other reverse genetic approaches. This method has proven to be successful to rapidly identify variant genotypes and determine gene function in plants that are diploid and have relatively small genomes such as *Arabidopsis*. Additionally, it also can be easily applied to other crop plants with very large genomes that are further complicated by various ploidy levels such as wheat. Moreover, the use of chemical mutagens in diploid and polyploid plants produce a range of various mutations and a high density of mutations throughout the genome. Obtaining an allelic series for a gene of interest greatly assists in the overall determination of gene function by providing multiple types of phenotypic variants to be analyzed.

Conclusion :

TILLING is a non-transgenic, high throughput reverse genetic approach. This technique unlike other SNP detection methods, provides the approximate location within a few base pairs of the induced mutation, which allows targeted sequencing in the area of the induced mutation opposed to sequencing the entire fragment. Chemical mutagensis produces a range of various mutations throughout the genome such as nonsense, splice site and missense, all of which potentially can affect the protein structure and the resulting phenotype, it has been used for decades

to obtain mutants for genetic studies. Therefore, through mutagensis one can obtain partial loss or complete loss of function and new novel functions, which can provide valuable insight into the true role of a gene in a species of interest. By using chemical mutagensis and TILLING to pinpoint these mutations has been highly effective in the elucidation of gene function in plants without the production of transgenic material. TILLING has been demonstrated to be sensitive enough to detect induced mutations and naturally occurring SNPs, as well as the detection of heterozygotes. EcoTILLING which has been less frequently employed in the current literature, can also be a valuable tool for mining for SNPs in germplasm, assessing heterozygosity, uncovering variants for disease resistance or ascertaining the function of a gene or regulatory element by detecting natural variants. EcoTILLING can be a good technique to employ especially when working with a well established population with thoroughly characterized morphological data.

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