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Review

Discovery of mutations with TILLING and ECOTILLING in plant genomes

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TILLING (Targeting Induced Local Lesions IN Genomes) and ECOTILLING are methods used in detecting induced or naturally occurring mutations in many species. High-throughput TILLING allows the rapid, easy and cost-effective discovery of induced point mutations in populations of chemically mutagenized individuals. The use of the TILLING technique to survey natural variation in genes is called ECOTILLING. TILLING and ECOTILLING have recently been used for the detection of both induced mutation and natural DNA polymorphism. In this review, we illustrate how TILLING and ECOTILLING methods can be employed for discovering mutations.

Key words: TILLING, ECOTILLING, mutation, natural.

INTRODUCTION

Nucleotide sequence variation is a major determinant of heritable phenotypic difference in plant genomes. Variation can either be natural, from divergent populations, or induced through treatment with mutagens (Till et al., 2007a). There are several methods used in discovery mutations, which are natural or induced through treatment with mutagens in the genomes.

TILLING and ECOTILLING are closely related methods that are useful in the rapid detection of small mutations and natural polymorphisms, respectively. TILLING (Targeting Induced Local Lesions IN Genomes) allows the identification of single-base-pair (bp) allelic variation of target gene in a high-throughput manner. The use of the TILLING technique to survey natural variation in genes is called ECOTILLING (Till et al., 2004; Comai et al., 2004). TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetic technique that is suitable for most plants (McCallum et al., 2000a). For TILLING, mutations are created by treatment with the same chemical mutagens that have been successfully employed in mutation breeding programs for decades. By using chemical mutagens that induce primarily random point mutations at high density, allelic series of missense and truncation mutations can be discovered with TILLING (Greene et al., 2003).

Several chemical mutagens can be used to create random point mutations. Ethylmethanesulfonate (EMS), an alkylating agent, is particularly effective because it forms adducts with nucleotides, causing them to impair with complementary bases, thus introducing base changes after replication (Haughn and Somerville, 1987; Ashburner, 1990). EMS alkylates guanine bases and leads to mispairing: Alkylated G pairs with T instead of C; thus, the resulting mutations are G/C-to-A/T transitions (Henikoff and Comai, 2003). Other alkylating agents such as ethylnitrosourea (ENU) have also been used to effectively induce non-specific mutations (Lightner and Caspar, 1998). The ideal mutagen for TILLING is the one that randomly induces single nucleotide substitutions, or small insertions/deletions (□<30 nucleotides) at a high frequency (Till at al., 2007a).

How is TILLING done?

TILLING consists of several major steps: Development of a mutagenized population, DNA preparation and pooling, and mutation discovery (Figure 1).

At first, random mutations are induced in genomes by using chemical mutagens, seeds are mutagenized by

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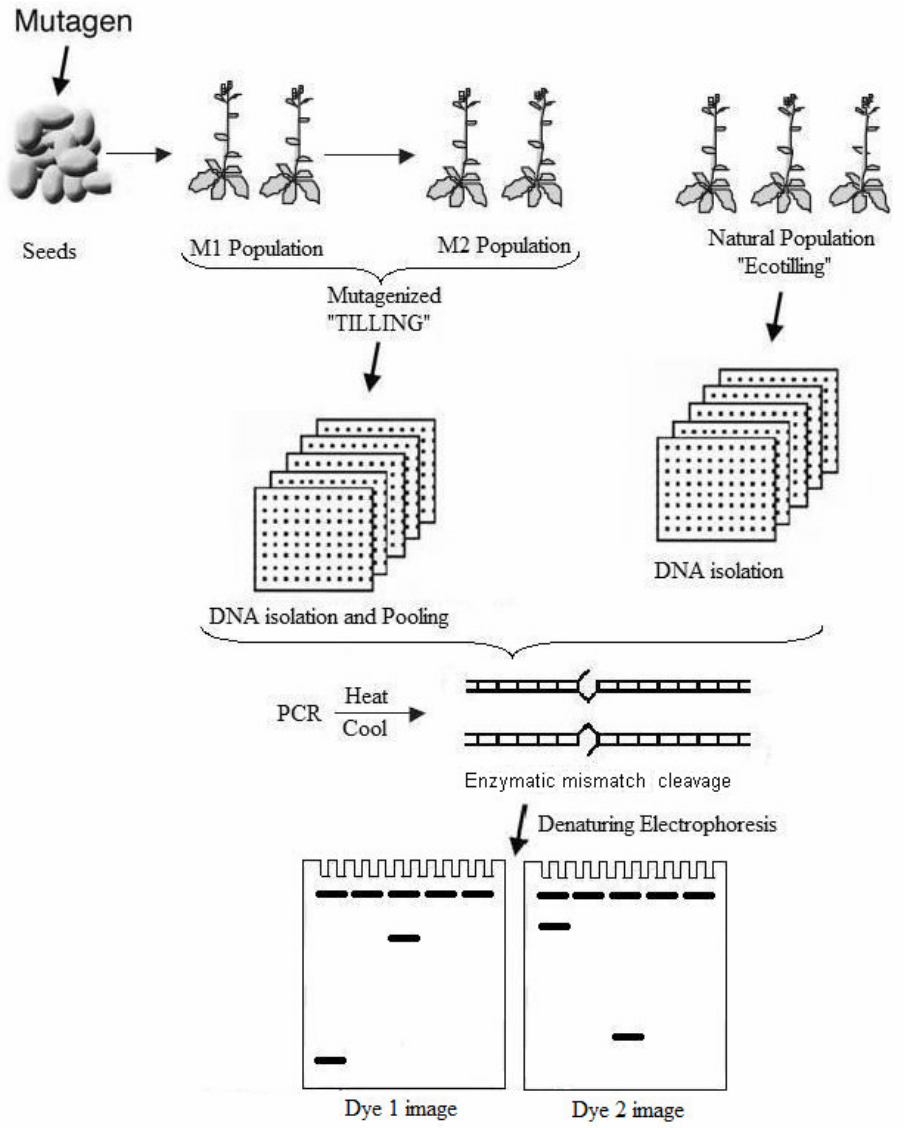

Figure 1. Outline of the basic steps for typical TILLING and EcoTILLING assays. Seeds are mutagenized by using chemical mutagens. The resulting M1 plants are self-fertilized, and M2 individuals are used to prepare DNA samples for mutational screening. DNA is collected from a mutagenized population (TILLING), or a natural population (EcoTILLING). For TILLING, DNAs are pooled. Typical EcoTILLING assays do not use sample pooling, but pooling has been used to discover rare natural single-nucleotide changes (Till et al., 2006). After extraction and pooling, samples are typically arrayed into a 96-well format. The target region is amplified by PCR with gene-specific primers that are end-labeled with fluorescent dyes. Following PCR, samples are denatured and annealed to form heteroduplexes that become the substrate for enzymatic mismatch cleavage. Cleaved bands representing mutations or polymorphisms are visualized using denaturing polyacrylamide gel electrophoresis.

treatment with ethylmethanesulfonate (EMS). The resulting M1 plants are self-fertilized, and M2 individuals are used to prepare DNA samples for mutational screening. DNA is extracted from test samples. The DNA samples are pooled and arrayed into microtiter plates. Screening for mutations begins with PCR amplification of a target fragment of up to 1.5 kb using gene-specific infrared dye-labeled primers. The forward primer is 5'-end labeled with a fluorescent dye that is detected at 700 nm (IRDye 700) and the reverse primer is labeled with the IRDye 800 nm (Till et al., 2006). These PCR products are denatured and reannealed to allow the formation of mismatches, or heteroduplexes, which represent naturally occurring single nucleotide polymorphisms

(SNPs) and induced SNPs. Samples, are then incubated with a single-strand specific nuclease to digest mismatched base pairs. For mismatch-specific cleavage, several enzymes, including S1 nuclease (Howard et al., 1999) and T4 endonuclease VII (Youil et al., 1996) have been used. After cleavaging, DNA samples are purified from buffer components and then each sample is loaded onto a denaturing polyacrylamide gel. Cleaved heteroduplexes produced two smaller molecular weight products, one labeled with IRDye 700 and the other with IRDye 800, whose sizes added up to the size of the full length product (Till, et al., 2007a). All of these processes are presented in Figure 1.

TILLING applications in plants

TILLING was first applied to *Arabidopsis thaliana* (McCallum et al., 2000ap; McCallum et al., 2000b). A mutagenized population was created by treating seeds with EMS. Proof of concept was shown by the discovery of novel alleles in two cytosine methyltransferase genes. These improvements allowed the creation of the first public TILLING service known as the Arabidopsis TILLING Project. The Arabidopsis TILLING project (ATP) was established in August 2001, giving the international research community the access to induced point mutations in *A. thaliana* (Till et al., 2003). ATP researchers have also developed web based software programs to calculate the putative effect of induced or natural polymorphisms on gene function. CODDLE (http://www.proweb.org/input) allows requestors to specifically design their PCR primers to target the functional domain in which they are interested or to target the most-conserved domain, which is likely to be the most sensitive to amino-acid substitutions (Gilchrist and Haughn, 2005). Also, the conservation-based SIFT (Sorting Intolerant from Tolerant) software predicts with approximately 75% accuracy, whether or not an amino acid change is damaging a protein (Ng and Henikoff, 2003). Also, the PARSESNP (for Project Aligned Related Sequences and Evaluate SNP's; http://www.proweb.org/parsesnp) program allows the user to input any number of nucleotide changes of a gene. By using a reference DNA sequence, an exon/intron position model and a list of polymorphisms, software reports the effects of these polymorphisms on the expressed gene product in a graphical format (Taylor and Greene, 2003).

Perry et al. (2003) adapted the TILLING method for the model legume *Lotus japonicus*, since June 2003; the *L. japonicus* TILLING facility has been available for research community to identify plants carrying point mutations for any gene of interest. In a pilot experiment, the frequency of point mutations was analyzed in the symbiosis defective (symbiosis receptor kinase) gene, which is required for root symbioses (Stracke et al., (2002) Using this population, 17 mutations were identified that relate to six independent alleles, thus demonstrating prof of concept (Perry et al., 2003)

The applicability of TILLING in a polyploid species for wheat was reported by Slade and Knauf (2005). Over 200 mutations were discovered in the pilot screen and the estimated mutation densities were exceptionally high: 1 mutation / 40kb in tetraploid and 1/24 kb in hexaploid wheat.

The TILLING method was applied to model crop rice (Till et al., 2007b). Two different mutagenic treatments provide a suitably high density of mutations (≥ 1/500 kb) to consider development of rice for a high throughput TILLING service. From this pilot-scale experiment with 10 target genes, 57 nucleotide changes were identified most of which were inferred to be induced by mutagen treatment. One nonsense mutation and 29 missense mutations were identified, six of which are predicted to cause damage to protein function (Till et al., 2007b).

It was shown that high-throughput TILLING is feasible to maize (an important commercial crop plant with a large genome but with limited reverse-genetic resources). Screening results from the pools of DNA samples for mutations in 1-kb segments from 11 different genes, obtaining 17 independent induced mutations from a population of 750 pollen mutagenized in maize plants. One of the genes targeted was the DMT102 chromomethylase gene, in which an allelic series of three missense mutations were obtained and are predicted to be strongly deleterious (Till et al., 2004).

ECOTILLING

Allowing of forceful discovery of mutations, high-throughput TILLING technology is ideal for the detection of natural polymorphisms: CEL I cuts with partial efficiency, allowing the display of multiple mismatches in a DNA duplex. Therefore, interrogating an unknown homologous DNA by heteroduplexing to a known sequence, reveals the number and position of polymorphic sites. Both nucleotide changes and small insertions and deletions are identified, including at least some repeat number polymorphisms. This method is called ECOTILLING. As with TILLING, ECOTILLING is general, and should be applicable to most species. The ECOTILLING allows the rapid detection of variation in many individuals and is cost effective because only one individual for each haplotype need to be sequenced. The technology is applicable to any organism including those that are heterozygous and polyploid (Comai et al., 2004).

The ECOTILLING method was used to detect variation in five gene target regions in 196 different *Arabidopsis* ecotypes (Comai et al., 2004). Based on this work, the modification of TILLING for the discovery and genotyping of natural nucleotide polymorphisms was termed ECOTILLING.Genotyping analysis of western black cottonwood populations (*Populus trichocarpa*) was

reported using ECOTILLING for SNP identification (Gilchrist et al., 2006). Sixty-three novel SNPs were identified in 9 target genes, for 41 tree accessions.

The ECOTILLING method also was applied to sugarcane (*Saccharum* sp.), a complex polyploid species, as a model to develop and test new protocols for high throughput ECOTILLING using capillary electrophoresis (Eliott et al., 2008).

CONCLUSIONS

Nucleotide sequence changes are very important element in view of heritable phenotypic difference in plant genomes. The changes of nucleotides bring about mutations in genomes like SNP(s) (Single Nucleotide Polymorphism), deletion(s) or insertion(s). These mutations can be either nonsense mutation or sense mutation. TILLING and ECOTILLING are related methods that are useful in the rapid and cheap detection of small mutations or natural polymorphisms. Mutations can also be discovered in several other ways like denaturing HPLC (Underhill et al., 1997), SSCP (Gross et al., 1999); however, these methods have not been shown to detect all types of mismatches in different sequence context. Another method is RFLP (Restriction Fragment-Length Polymorphism), which is the oldest but still a useful marker system in the determination of mutations and to research allelic diversity in plants (Simsek, 2009). RFLP has several disadvantages in comparison with TILLING and ECOTILLING; in fact RFLP is not a rapid and automated technique and therefore, requires radioactive materials. On the contrary, TILLING and ECOTILLING have several advantages, since mutations can be detected easily and these techniques can be used instead of full sequencing.

This mutation screening method can be applied to several plant species whether small or large, diploid or allohexaploid and may provide a rapid approach to reverse genetics by the identification of induced and naturally occurring variation in many plant species.

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