TILLING (Targetting Induced Local Lesions In Genomes) Technology for Plant Functional Genomics

R. Soner SİLME M. İlhan ÇAĞIRGAN

Department of Field Crops, Faculty of Agriculture, Akdeniz University, Antalya, TURKEY
e. mail: ragipsoner@Akdeniz.edu.tr

Received : 07 July 2006
Accepted : 16 October 2006

Abstract

The present review describes TILLING (Targetting Induced Local Lesions In Genomes) which is a general reverse-genetic strategy that works with a mismatch-specific endonuclease to detect induced or natural DNA polymorphisms in genes of interest. With high-throughput TILLING, rapid and low-cost discovery of induced point mutations in populations of chemically mutagenized individuals from diverse organisms becomes possible. Other advantages are its independence of genome size, reproductive system or generation time. TILLING yields a traditional allelic series of point mutations which make it valuable for essential genes, where sublethal alleles are required for phenotypic analysis. Current advantages of TILLING have made it an appropriate choice for the detection of both induced and natural variation in several plant species. Here, we review recent progress in this technology for the researchers of plant mutation analysis and genomics era.

Keywords: chemical mutagenesis, reverse genetics, single-nucleotide polymorphism discovery, allelic series

INTRODUCTION

The ability to induce mutations has been used by geneticists for the past 75 years [1,2]. 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 [3,4]. Furthermore, the effect of EMS mutagenesis often results in a large number of recessive mutations across the genome [5]. Other alkylating agents such, as ethylnitrosourea (ENU), have also been used to effectively induce non-specific mutations [5]. 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 function-studies. 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 [6]. 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.

How TILLING Works

Basic TILLING method allows for high-throughput identification of single-base-pair (bp) allelic variations [7]. The first step starts with mutagenized seeds obtained from treatment with EMS. The resulting M1 plants are self-fertilized and the M2 generation of individuals is used to prepare DNA samples for mutational screening. The DNA samples are pooled, arrayed on microtiter plates and subjected to gene-spesific PCR [8]. Amplification products are incubated with an endonuclease such as CELI, a member of the S1 nuclease family of single strand-specific nucleases [9]. CELI cleaves the 3' side of mismatched DNA where the heteroduplex between the wild-type and the mutant strands of DNA loops out; homoduplexes are left intact [10]. Cleavage products are electrophoresed using an automated sequencing gel apparatus, and gel images are analyzed by examining the gel readout with the aid of a standard commercial image-processing program. Differential double-end labeling of amplification products allows for rapid visual confirmation because mutations are detected on complementary strands and can be easily distinguished from amplification artifacts.

Upon detection of a mutation in a pool, the individual DNA samples are similarly screened to identify the plant carrying the mutation. This rapid screening procedure determines the location of a mutation to within ±10 bp for PCR products that are 1-kb in size [25].

For mismatch-specific cleavage, several enzymes, including S1 nuclease [11,12] and T4 endonuclease VII [13] have been used. CELI, a plant-specific extracellular glycoprotein, has been shown to be suitable for genotyping applications because it preferentially cleaves mismatches of all types [9] and has been used to detect heterozygous polymorphisms in DNA pools [10]. Further research showed that comparison of CELI with other single-strand specific nucleases revealed the endonuclease activity does not differ between these enzymes under optimized conditions [12]. Also, plant extracts performed as well as the highly purified preparations. These results suggest that something in the crude sample either enhances the endonuclease activity of these enzymes at heteroduplex sites or inhibits their activity at the amplicon ends.

In brief, screening is performed on DNAs that have been arrayed in 96-well microtiter plates and pooled eightfold to maximize screening efficiency. Gene specific, fluorescently-tagged primers are used to amplify pooled DNA (Figure 1).

The amplification products are denatured and allowed to reanneal, generally by heating and cooling. As a result, a mutant strand will often reanneal with a wild-type strand, creating heteroduplexes at the site of the mutation or polymorphism. The resultant double-stranded products are digested with CELI, which cleaves one of the two strands at the heteroduplex mismatches. Cleaved products, which are detected on polyacrylamide denaturing gels, identify individuals that have a mutation in the gene of interest. The size of the fragments carrying the 5' and 3' fluorescent tags can be used to estimate the position of the mutation within the amplicon.

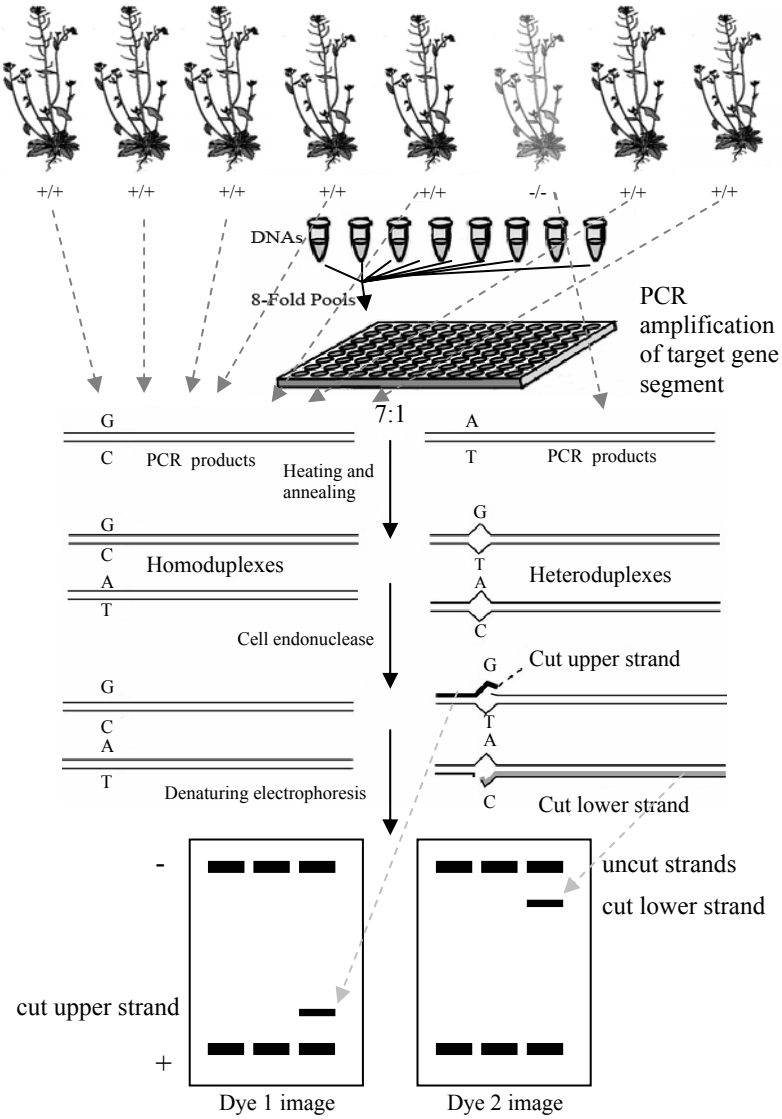

Figure 1. Schematic diagram of TILLING method is redrawn with permission from the Annual Review of Plant Biology, (Volume 54 (c)2003 by Annual Reviews, www.annualreviews.org) from Henikoff and Comai, 2003 [14]. In each of 96 wells of a plate, the DNA from eight plants are pooled. During the amplification of pooled DNA fluorescently tagged gene-specific primers are used. The amplified products are denaturated by heating and cooling slowly for randomly re-annealing and forming homo- and heteroduplexes. Before denaturation is performed, the resultant double-stranded products are digested by CELI endonuclease. EMS treatment of seeds results alkylation of guanine bases and leads to mispairing such as G pairs with T instead of C. CELI cuts mismatched sites resulting shorter strands detectable differentially on a polyacrylamide gel, asymmetricly labeling with infrared dyes from 5' and 3' sites [14]

TILLING projects in different species

Many TILLING projects are ongoing in diverse plant species, such as *Arabidopsis* [7,15,16], *Brassica oleracea* [17], lotus [18], maize [19], barley [20], wheat [21] and some animal species such as rat [22] and zebrafish [23, 24].

In 2001, *Arabidopsis* TILLING Project (ATP) started with cooperation of the Comai Laboratory at University of Washington and the Henikoff Laboratory at the Fred Hutchinson Cancer Research Center (Seattle, WA) [7,25]. As a result of this cooperation, scientists are able to for search mutations in genes of interest. After a search for mutations is initiated, approximately 10 mutations are delivered within 2 to 3 months. Among these, the scientists would have a high probability of finding hypomorphic alleles. If this does not suffice, then all the available TILLING lines (approximately 7,000) could be searched, which would provide approximately 25 different point mutations, half of which, on average, would be missense [25].

ATP researchers also have been developed or adapted several computer programs to facilitate the TILLING process in Bioinformatics. One of these softwares, CODDLE, is a web-based software for submitting a genomic sequence and for obtaining an exon-intron model for the gene of interest using public sequence databanks. CODDLE (http://www.proweb.org/input) allows researchers to specifically design PCR primers to target the functional domain in which they are interested or to target the most-conserved domain, which is likely sensitive to amino-acid substitutions. Also, the conservation-based SIFT (Sorting Intolerant From Tolerant) program predicts with approximately 75% accuracy whether or not an amino acid change is damaging to a protein [26]. 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, program reports the effects of these polymorphisms on the expressed gene product in a graphical format as a result. [27].

At John Innes Centre in the UK, a TILLING project for the legume *Lotus japonicus* has also been started. The aim of this project is screening a library of DNA from plants that have been identified as symbiosis-defective. Until recently, no suitable insertional mutagenesis tool is developed for *L. japonicus* and the research community is hoping TILLING technology may solve this problem by generating an initial series of genetic mutations. Further information for the mutant plants is available at http://www.lotusjaponicus. org/finder.htm, and this web-site provides a valuable resource for the *Lotus* community [18].

Similar results are taken from the TILLING project of maize (http://genome.purdue.edu/maizetilling; discovery of maize TILLING) to that in *Arabidopsis*. These results show that high-throughput TILLING is applicable to maize, an important commercial crop plant with a large genome but with limited reverse-genetic resources [19]. 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 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.

There are current projects in different organisms such as the barley TILLING project at the University of Bologna-ITALY, Department of Agroenvironmental Science and Technology (DiSTA) by Talamè and colleagues [20]. Also, Slade and colleagues [21] mutagenized (allohexaploid and allotetraploid) wheat strains with EMS and identified 246 alleles of the waxy genes in 1920 individuals. As a result of this project, a line of bread wheat which contains a homozygous mutation in the two created waxy homeologs and a preexisting deletion of the third waxy homeolog, displays a near-null waxy phenotype.

ECOTILLING

In addition to allowing efficient detection of mutations, TILLING technology is also ideal for examining natural as well as induced variation. Endonuclease CELI cuts with partial efficiency making possible to display multiple mismatches in a DNA duplex. Therefore, heteroduplexing DNA of unknown sequence with to that of a known sequence reveals the positions of polymorphic sites. Both nucleotide changes and small insertions/deletions are identified, as well as some repeat number polymorphisms. This method, called EcoTilling by Henikoff and Comai, was used to examine variation in five genes in 96 different *Arabidopsis* accessions [16] and Gilchrist and colleagues used this method to to survey genetic variation of *Populus trichocarpa* [28].

Ecotilling can be performed more inexpensive than full sequencing, the method currently used for most single nucleotide polymorphism (SNP) discovery [25]. SNP variation can provide clues to the adaptive strategies and population history that undoubtedly played roles in species evolution. Also, industrial EcoTilling is used by Anawah Inc. (http://www.anawah.com) for screening and detection of plants with desired traits by knockdown and knockout mutations in specific genes.

CONCLUSIONS

Nucleotide sequences contain hidden information about the forces for conservation and variation that shaped their evolutionary history. The genes that control the function of the biological mechanisms involved are in many cases identified and well characterised. It is known that detection of a gene responsible for a mutation is a challenging process. TILLING, a cheap and fast natural polymorphism discovery and genotyping method has advantages for determining the spectrum of variation and for genetic mapping based on linkage association analysis.

In TILLING technique, if a mutation is detected in a pool, the individual DNA samples that went into the pool can be individually analyzed to identify the individual that carries the mutation. Once this individual has been identified, its phenotype can be determined. This technique works with good results even if a population contains pre-existing mutations that would compromise SNP discovery by other methodologies [29].

In this review, we illustrate how chemical mutagenesis is becoming a powerful tool especially for reverse genetics in plant species by using the TILLING approach. The use of TILLING can facilitate the handling of the large-scale discovery of induced point mutations through populations that are required. This new 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 identification of induced and naturally occurring variation in many species. When commercially suitable variations are discovered TILLING has the advantage of been exempt from regulatory approval requirements which is strictly obliged for transgenic crops. These properties make TILLING approach a valuable tool for mutation analysis.

REFERENCES

[1]. Muller HJ. 1930. Types of visible variations induced by X-rays in Drosophila. Journal of Genetics. 22: 299-334.

[2]. Stadler LJ. 1932. On the genetic nature of induced mutations in plants. Proceedings of the VI Congress of Genetics. 1:274-294.

[3]. Haughn G and Somerville CR. 1987. Selection for herbicide resistance at the whole-plant level, pp.98-107 in Applications of Biotechnology to Agricultural Chemistry, edited by H.M. Lebaron, R.O. Mumma, R.C. Honeycutt and J.H. Duesing. American Chemical Society, Easton, PA.

[4]. Ashburner M. 1990. Drosophila: A Laboratory Handbook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[5]. Lightner J, Caspar T. 1998. In J Martinez-Zapater, J Salinas, eds, Methods on Molecular Biology, Vol 82. Humana Press, Totowa, NJ, pp 91-104.
[6]. Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, et al. 2003. Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. Genetics. 164:731-740.
[7]. Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Young K, Taylor NE et al. 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Research. 13:524-530.
[8]. Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S. 2001. High-throughput screening for induced point mutations. Plant Physiology. 126:480-484.
[9]. Oleykowski CA, Mullins CRB, Godwin AK, Yeung AT. 1998. Mutation detection using a novel plant endonuclease. Nucleic Acids Research. 26:4597-4602.
[10]. Kulinski, J, Besack, D, Oleykowski, CA, Godwin, AK and Yeung, AT. 2000. CEL I enymatic mutation detection assay. Biotechniques. 29:44-46.
[11]. Howard, JT, Ward, J, Watson, JN and Roux, KH. 1999. Heteroduplex cleavage analysis using S1 nuclease. Biotechniques, 27, 18-19.
[12]. Till BJ, Burtner C, Comai L, Henikoff S. 2004. Mismatch cleavage by single- strand specific nucleases. Nucleic Acids Research. 32:2632-2641.
[13]. Youil, R, Kemper, B, and Cotton, RGH. 1996. Detection of 81 of 81 known mouse b-globin promoter mutations with T4 endonuclease VII - The EMC method. Genomics 32:431-435.
[14]. Henikoff S and Comai L. 2003. Single-nucleotide mutations for plant functional genomics. Annual Reviews of Plant Biology. 54:375-401.
[15]. McCallum CM, Comai L, Greene EA, Henikoff S. 2000. Targeted screening for induced mutations. Nature Biotechnology. 18:455-457.
[16]. Comai L, Young K, Reynolds SH, Codomo C, Enns L, Johnson J, Burtner C, Henikoff JG, Greene EA, Till BJ, Henikoff S. 2004. Efficient discovery of nucleotide polymorphisms in populations by ecotilling. Plant Journal. 37:778-786.
[17]. Gilchrist EJ and Haughn GW. 2005. TILLING without a plough: a new method with applications for reverse genetics. Current Opinion in Plant Biology. 8:211–215.
[18]. Perry JA, Wang TL, Welham TJ, Gardner S, Pike JM, Yoshida S, Parniske M. 2003. A TILLING reverse genetics tool and a webaccessible collection of mutants of the legume Lotus japonica. Plant Physiology. 131:866-871.
[19]. Till BJ, Reynolds SH, Weil C, Springer N, Burtner C, Young K, Bowers E, Codomo CA, Enns LC, Odden AR et al. 2004. Discovery of induced point mutations in maize genes by TILLING. BioMedCentral Plant Biology. 4:12.
[20]. Talamè V, Salvi S, Sanguineti MC, Tuberosa R. 2005. Development of a TILLING Reverse Genetics Resource in Barley. Proceedings of the Plant & Animal Genomes XIII Conference. 81.
[21]. Slade AJ, Fuerstenberg SI, Loeffler D, Steine MN, Facciotti D. 2005. A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. Nature Biotechnology. 23:75-81.
[22]. Smits BMG, Mudde J, Plasterk RHA, and Cuppen E 2004. Target-selected mutagenesis of the rat. Genomics. 83:332–334.
[23]. Wienholds E, Van Eeden F, Kosters M, Mudde J, Plasterk RH, Cuppen E. 2003. Efficient target-selected mutagenesis in zebrafish. Genome Research. 13:2700-2707.
[24]. Sood R, English MA, Jones M, Mullikin J, Wang D, Anderson M, Wu D, Chandrasekharappa SC, Yu J, Zhang J and Liu PP. 2006. Methods for reverse genetic screening in zebrafish by resequencing and TILLING. Methods. 39:220-227.
[25]. Henikoff S, Till BJ, Comai L. 2004. TILLING. Traditional mutagenesis meets functional genomics. Plant Physiology. 135:630-636.
[26]. Ng PC and Henikoff S. 2003. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Research. 31:3812-3814.
[27]. Taylor NE and Grene EA. 2003. PARSESNP: a tool for the analysis of nucleotide polymorphisms. Nucleic Acids Research. 31: 3808–3811.
[28]. Gilchrist EJ, Haughn GW, Ying CC, Otto SP, Zhuang J, Cheung D, Hamberger B, Aboutorabi F, Kalynyak T, Johnson L, Bohlmann J, Ellis BE, Douglas CJ, Cronk QCB. 2006. Use of Ecotilling as an efficient SNP discovery tool to survey genetic variation in wild populations of *Populus trichocarpa.* Molecular Ecology. 15:1367-1378.
[29]. Slade AJ and Knauf VC. 2005. TILLING moves beyond functional genomics into crop improvement. Transgenic Research. 14:109–115.