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# TILLING without a plough: a new method with applications for reverse genetics

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TILLING (Targeting Induced Local Lesions IN Genomes) is a powerful reverse genetic technique that employs a mismatch-specific endonuclease to detect induced or natural DNA polymorphisms. Its advantages over other reverse genetic techniques include its applicability to virtually any organism, its facility for high-throughput and its independence of genome size, reproductive system or generation time. TILLING is currently being used for the detection of both induced and natural variation in several plant species.

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## Introduction

Genome sequencing projects have been completed for several plant species and many more plant genome projects are underway. To exploit the potential of this resource, researchers have shifted their attention to a variety of functional genomics tools that can help decipher the functions of the thousands of newly identified genes.

Genetic mutation is a powerful tool that establishes a direct link between the biochemical function of a gene product and its role *in vivo*. For decades, genes have been identified and the function of their products determined by isolating and studying mutants that are defective in a specific process of interest (i.e. by forward genetics). With the advent of molecular biology, several distinct techniques have been developed to generate or identify mutations in cloned genes to determine the function of these genes (reverse genetics). In plants, the most commonly used reverse genetic approaches are posttranscriptional gene silencing (PTGS) [1] and insertional mutagenesis

[2]. Such methods have several disadvantages, however, that limit their use for functional genomics. PTGS is labour-intensive, can give ambiguous results, and is unsuitable for isolating mutants that have lethal or sterile phenotypes. For insertional mutagenesis, the frequency of mutations per genome is typically low, necessitating the screening of large numbers of plants in a mutagenised population to identify mutations in any given gene. In addition, the mutant alleles are likely to result in a complete loss-of-function for the gene product which, if the effect is lethal or detrimental, might limit the analysis that can be done.

The use of chemical mutagens represents an alternative method of mutagenesis that has none of the drawbacks of the methods mentioned above. Ethylmethane sulfonate (EMS) has been the most commonly used mutagen in plants and induces large numbers of recessive mutations per genome [3]. Other alkylating agents such as ethylnitrosourea (ENU) have also been used effectively. Induced point mutations can create a diverse range of alleles for genetic analysis. In *Arabidopsis*, five percent of EMS-induced mutations in targeted coding regions result in premature termination of the gene product, whereas fifty percent result in missense mutations that alter the amino-acid sequence of the encoded protein [4]. Other advantages of EMS mutagenesis are that the mutations generated are randomly distributed in the genome, and that a high degree of mutational saturation can be achieved without excessive collateral DNA damage. Despite the clear advantages of EMS mutagenesis, until recently, it has not been useful as a tool for reverse genetics because of the lack of high-throughput techniques for detecting point mutations.

## The TILLING technique

TILLING (Targeting Induced Local Lesions IN Genomes) allows the identification of single-base-pair (bp) allelic variation in a target gene in a high-throughput manner [5]. It has several advantages over other techniques used to detect single-bp polymorphisms. Other gel-based assays, such as single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE), do not pinpoint the location or the type of polymorphism present in the DNA fragment [6]. Techniques that rely on denaturation kinetics can be observed with quantitative polymerase chain reaction (PCR) but only work for small fragments of DNA [7], and array hybridization techniques are only effective in discovering approximately 50% of simple nucleotide polymorphisms (SNPs) [8].

Originally, the TILLING technique used denaturing high performance liquid chromatography (HPLC) to detect mismatches in heteroduplex DNA that had been generated by PCR amplification from a pooled population of wildtype and mutant *Arabidopsis* plants [9,10]. A less-expensive and faster modification of the TILLING protocol was published by Colbert *et al.* [11] and employs a mismatch-specific celery nuclease, CEL1, to identify SNPs [12]. A subsequent comparison of this nuclease with other single-strand specific (sss) nucleases by Till and colleagues [13\*\*] revealed that the endonuclease activity of the celery enzyme does not differ significantly from that of *Aspergillus* S1 nuclease or from mung bean nuclease if the digestion conditions are optimised for each enzyme. Interestingly, Till and colleagues [13\*\*] also found that crude juice extracts appear to perform as well as, if not better than, the purified nucleases for analysis of SNPs in wild populations. This suggests that something in the crude extract either enhances the endonuclease activity of these enzymes at heteroduplex sites or inhibits their activity at the ends of the amplicons where cleavage of the fluorescent tags would lead to loss of detection.

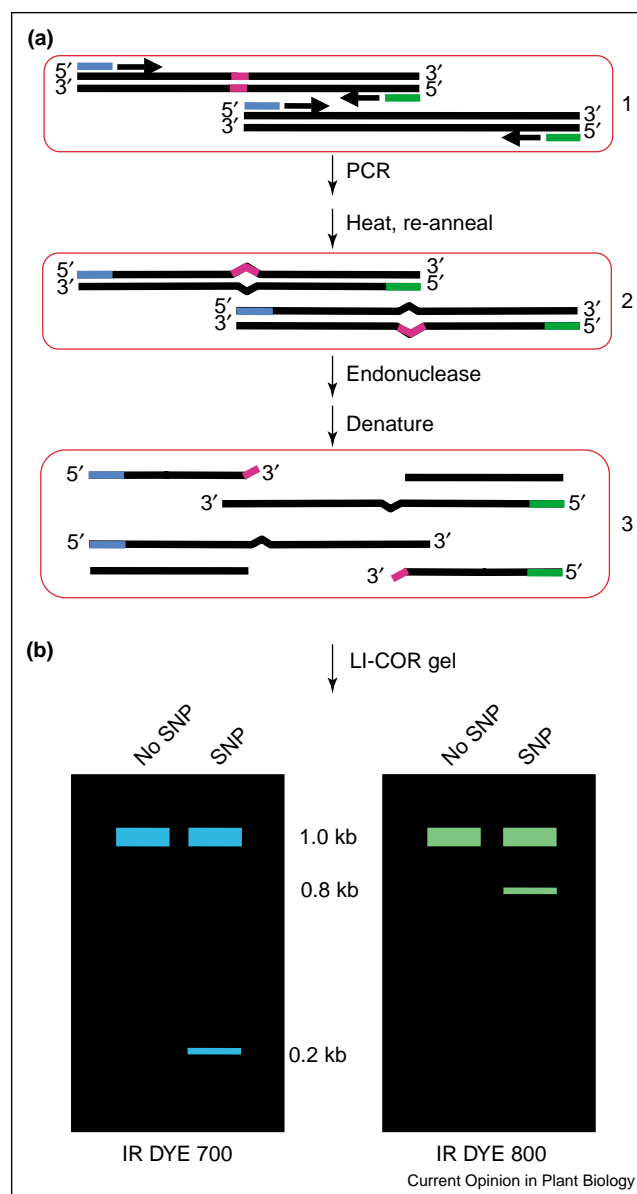
Figure 1 shows an overview of the TILLING procedure. Briefly, pooled DNA is amplified using fluorescently tagged, gene-specific primers. The amplified products are denatured by heating and then allowed to cool slowly so that they randomly re-anneal. Heteroduplex molecules form when a pool includes at least one plant with a mutation in the amplified region. The resultant double-stranded products are digested with a sss nuclease, which cleaves one of the two strands at heteroduplex mismatches. Cleaved fragments, which are detected on polyacrylamide denaturing gels, identify individuals that have a mutation in the gene of interest. The position of the mutation within the amplicon can be estimated from the size of the fragments carrying the 3' and 5' fluorescent tags. The LI-COR system (LI-COR Biosciences, Lincoln, NE) is currently used for most TILLING projects because of the sensitivity of the LI-COR laser detectors with their fluorophors, but the potential savings in time and gel costs that would result from the use of a capillary detection system make the development of this possibility a desirable goal for the future.

### TILLING projects in different species

Several TILLING reverse genetics projects are now underway in diverse plant species, including *Arabidopsis* [5\*\*], lotus [14], maize [15] and *Brassica oleracea* (E) Gilchrist, I Parkin, GW Haughn, unpublished), and in some animal species [16–19]. Further discussion of the latter is beyond the scope of this review.

In 2001, Till and colleagues [5\*\*,20,21] established the *Arabidopsis* TILLING Project (ATP) as a joint effort between the Comai Laboratory at the University of Washington and the Henikoff Laboratory at the Fred

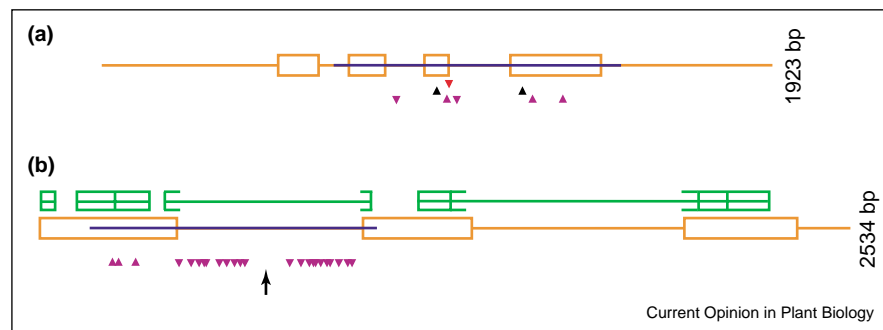
Figure 1



The TILLING procedure. (a) (1) Pooled DNA is amplified using fluorescently tagged gene-specific primers. (2) The amplified products are denatured by heating and then allowed to cool slowly so that they randomly re-anneal. (3) The resultant double-stranded products are digested with a sss nuclease and then denatured. (b) Fragments are then detected on a polyacrylamide gel, thus identifying DNA populations that carry a mutation in the gene of interest. The LI-COR gel system uses two channels to differentiate between the IRD700 and IRD800 dyes that label the 5' and 3' ends of the PCR fragment, respectively. The size of the cut fragments indicates the position of the mutation within the gene. In this case, the mutation is approximately 0.2 kb from the 5' end of the amplicon, and approximately 0.8 kb from the 3' end.

Hutchinson Cancer Research Center in Seattle, WA. They set up a facility that allows users to request mutations in genes of interest for a fee that covers part of the cost of providing this service. Typically, approximately 10

Figure 2



Graphic output from PARSESNP [22]. Variants in candidate genes were examined either by (a) TILLING or (b) Ecotilling. Blue lines that indicate the extent of the amplified region have been added to the PARSESNP-output figure. Orange open boxes denote exons. Purple arrowheads that point upwards indicate changes in the DNA sequence that do not affect the amino-acid sequence of the protein product. Purple arrowheads that point downwards indicate changes to non-coding regions of DNA. Black arrowheads that point upwards indicate changes that induce missense mutations in the protein product. Red arrowheads indicate changes that introduce a premature stop codon or splice-site error into the coding region of the gene. Green triple bars above the gene model indicate blocks of sequence that are conserved in homologous proteins. The vertical black arrow at the bottom of the figure indicates a relatively well conserved region within an intron.

mutations per gene are identified after screening a core library of ~3000 mutant *Arabidopsis* plant lines. Screening is continued, up to 6912 plants, if no deleterious mutations are found in the initial screen. Information on any mutations detected in the screens is released into the public domain within six months of providing the requestor with the sequences and accession numbers of their mutations, and all of the seeds from mutant stocks generated by the ATP are freely available from the *Arabidopsis* Biological Resource Center. An additional and very significant contribution that this group has made is in the popularisation of this technique through training sessions, workshops, and on-going support provided to researchers who are interested in developing TILLING in other organisms. 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. PARSESNP [22] allows the user to input any number of nucleotide changes in a gene and prints out the position of these polymorphisms in a graphical format (Figure 2), as well as indicating the change in the nucleotide and amino-acid sequences and documenting any restriction endonuclease sites that have been altered.

A TILLING facility for the legume *Lotus japonicus* has also been set up, by Perry and colleagues [14] at the John Innes Centre in the UK. Their initial approach was to screen a library of DNA from plants that had already been identified as being symbiosis-defective. It was reasoned that this tactic would allow a greater possibility of recovering useful alleles in genes of interest while reducing

the number of plants that it is necessary to screen. For *L. japonicus*, which has no insertional mutagenesis tool, this approach may be appropriate for generating an initial series of interesting mutations for the research community. The frequency of mutations recovered from this population was certainly higher than for an untargeted mutagenized population of the same size. However, results for only one gene were reported, and prior knowledge of the function of this gene was essential in the decision to screen for mutations in it. Details of the mutant plants are available at <http://www.lotusjaponicus.org/finder.htm>, and this information is itself a valuable resource for the *Lotus* community. More recently, the same group has developed, and arrayed for TILLING, an unscreened population of 5000 mutagenized *L. japonicus* plant lines.

The maize project is the most recent large-scale TILLING project to be established, and is expected to be open to the public by the end of 2004 (<http://genome.purdue.edu/maizetilling>; [15]). TILLING in maize has yielded similar results to that in *Arabidopsis*, even with a genome 20 times the size. The rate of recovery of mutations in maize is lower than that in *Arabidopsis*, necessitating the screening of more mutagenised plants to recover the same number of mutations per DNA segment (4000 individuals versus 2300 for *Arabidopsis*). Nevertheless, these data underscore the great potential of using TILLING for reverse genetics in many different organisms, regardless of genome size, reproductive system or generation time. In addition, considering current concerns about genetically modified crop plants, there is likely to be great interest in generating economically important variants without the need for genetic modifications that involve exogenous DNA. It has even been demonstrated recently that TILLING can be used for

reverse genetics in polyploid organisms [23]. Slade and colleagues [23] mutagenized both allohexaploid and allotetraploid strains of wheat with EMS and identified 246 new alleles of waxy locus homeologues in 1920 individuals screened. The authors successfully created one homozygous hexaploid line with a 'near-null' waxy phenotype using two of their newly identified alleles and a previously existing deletion of one of the homeologues.

### Ecotilling

TILLING technology holds much potential for examining natural as well as induced variation. Many of the species for which complete genome sequence data are available, including the tree *Populus trichocarpa*, are not amenable to classical mutagenesis and genetic analysis. For these organisms, the identification of natural genetic variants can provide much information about gene function, and can also be useful for association mapping and linkage disequilibrium analysis. Even in species for which considerable genetic resources are available, such as *Arabidopsis*, natural variation has allowed the elucidation of characteristics that would not have been identified using only induced mutations [24]. In addition, SNP variation can provide clues to the adaptive strategies and population history that play roles in species' evolution. Henikoff and Comai [25] coined the term 'Ecotilling' to refer to this use of the TILLING technique and, with their colleagues, they have used it to examine variation in five genes in 96 different *Arabidopsis* accessions [26••]. They were able to detect small deletions, insertions and microsatellite polymorphisms in addition to single-bp changes in the DNA sequence. Only the ends of the PCR product are labelled for TILLING and as there are often multiple polymorphisms within a single gene, the conditions of nuclease digestion used are such that only partial cleavage of the DNA is achieved. This results in a final collection of fragments that represent cleavage at each of the individual polymorphic sites within the amplicon.

With inbred species, such as *Arabidopsis*, that are mostly homozygous at any particular locus, the DNA from the different accessions must be pooled with a standard genotype because the endonuclease used for TILLING will only cleave at the site of a mismatch. With outbreeding species such as poplar, which are highly heterozygous, Ecotilling can be used not only to determine the extent of variation but also to assay the level of heterozygosity within a gene (EJ Gilchrist, GW Haughn *et al.*, unpublished). In addition, we speculate that by identifying blocks of conserved sequence within relatively unconserved non-coding regions, TILLING may help identify regulatory domains (Figure 2). One of the strengths of Ecotilling is that it significantly reduces the amount of sequencing that needs to be done to determine the nucleotide sequence of a gene in a large number of different individuals from a population. The sequence

from one or two representative samples will indicate what the DNA polymorphism is at any particular site in each of the carriers.

### Conclusions

TILLING is a technique that adds significantly to the arsenal of reverse genetics tools that are available to researchers wanting to capitalise on the information being provided by genome-sequencing projects. It is efficient and cost-effective, and both mutagenised and natural populations of any organism can be screened. As no reverse genetics technique developed to date is ideal for all purposes, TILLING complements other techniques well and, in the absence of site-directed mutagenesis in flowering plants, it is one of the few methods of detecting missense mutations in a high-throughput manner available to plant geneticists. The value of such missense mutations has long been recognized by classical genetics researchers as being essential for the elucidation of complex gene functions and gene interactions. TILLING competes easily with direct sequencing as a means of quickly identifying point mutations in genes of interest. Although improvements in pooled sequencing procedures or the development of novel mutagenesis techniques may eventually make TILLING obsolete, at present, it remains the technique of choice for medium-to-high throughput reverse genetics in many organisms.

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