

TILLING. Traditional Mutagenesis Meets Functional Genomics

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Most of the genes of an organism are known from sequence, but most of the phenotypes are obscure. Thus, reverse genetics has become an important goal for many biologists. However, reverse-genetic methodologies are not similarly applicable to all organisms. In the general strategy for reverse genetics that we call TILLING (for Targeting Induced Local Lesions in Genomes), traditional chemical mutagenesis is followed by high-throughput screening for point mutations. TILLING promises to be generally applicable. Furthermore, because TILLING does not involve transgenic modifications, it is attractive not only for functional genomics but also for agricultural applications. Here, we present an overview of the status of TILLING methodology, including Ecotilling, which entails detection of natural variation. We describe public TILLING efforts in *Arabidopsis* and other organisms, including maize (*Zea mays*) and zebrafish. We conclude that TILLING, a technology developed in plants, is rapidly being adopted in other systems.

Large-scale DNA sequencing projects have changed the way that biology is performed. The traditional pursuit of a gene starting with a phenotype has given way to the opposite situation: most of the genes are known from sequence, but most of the phenotypes are obscure. Thus, reverse genetics has become an important goal for many biologists, and new technologies are in great demand (Nagy et al., 2003). However, unlike genomic technologies such as DNA sequencing and BLAST searching, reverse-genetic methodologies are not similarly applicable to all organisms. For example, T-DNA insertional mutagenesis has turned the problem of obtaining a gene knockout into an *in silico* procedure for >70% of *Arabidopsis* genes (Alonso et al., 2003), but no comparable resources exist for rice (*Oryza sativa*) or maize (*Zea mays*), despite the increasing availability of high-coverage genomic sequence. RNAi-based silencing is an exciting strategy for reverse genetics (Waterhouse et al., 1998); however, throughput is limited by the difficulty of delivering siRNAs to target loci. Furthermore, the promise of using these reverse-genetic technologies for crop improvement is hampered by genetically modified organism issues.

Over the past few years, we and our colleagues have been developing a general strategy for reverse genetics that we call TILLING (for Targeting Induced Local Lesions in Genomes; McCallum et al., 2000). In TILLING, traditional chemical mutagenesis is followed by high-throughput screening for point mutations. Because of the wide use of chemical mutagenesis for forward-genetic screens in many organisms, TILLING promises to be generally applicable. TILLING is no different from traditional mutation breeding as far as the organism is concerned, so genetically modified

organism issues do not arise. This makes TILLING an attractive strategy not only for functional genomics, but also for agricultural applications.

The impetus for TILLING arose from a graduate student's frustration with the limitations of reverse-genetic methods available for *Arabidopsis* in the late 1990s. The student, Claire McCallum, went on to demonstrate the feasibility of TILLING by discovering mutations in two chromomethylase genes that were the subject of her thesis research (McCallum et al., 2000). Claire is currently Director of Research at Anawah, which performs industrial TILLING (<http://www.anawah.com>), while we have directed a National Science Foundation (NSF)-funded effort to improve and disseminate TILLING technology. Here, we present an overview of public TILLING efforts in *Arabidopsis* and other organisms.

HOW TILLING WORKS

The original TILLING method used a commercial denaturing HPLC (DHPLC) apparatus for mutation discovery. However, we anticipated that this method would not scale up easily, and so we looked at alternative technologies. A method for enzymatic mismatch cleavage described by Tony Yeung seemed particularly attractive (Oleykowski et al., 1998), so we proceeded to adapt it for high throughput. We found that the LI-COR gel analyzer system (Lincoln, NE; Middendorf et al., 1992) is ideally suited for this application. By mid-2001, we had established robust protocols and software to begin a TILLING production operation on our mutagenized *Arabidopsis* populations (Colbert et al., 2001).

For TILLING *Arabidopsis*, seeds are mutagenized by treatment with ethylmethanesulfonate (EMS). The resulting M1 plants are self-fertilized, and M2 individuals are used to prepare DNA samples for mutational screening, while their seeds are inventoried and

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sent to the Arabidopsis Biological Resource Center (ABRC) for eventual distribution. The DNA samples are pooled and arrayed in microtiter plates, and the pools are amplified using gene-specific primers. Amplification products are incubated with the CEL I endonuclease, a member of the S1 nuclease family of single strand-specific nucleases (Oleykowski et al., 1998). CEL I cleaves to the 3' side of mismatches and loop outs in heteroduplexes between wild-type and mutant DNA while leaving duplexes intact. Cleavage products are electrophoresed using the LI-COR gel analyzer system, and a standard commercial image processing program (Adobe Photoshop; Adobe Systems, Mountain View, CA) is used to examine the gel readout. Differential double-end labeling of amplification products allows for rapid visual confirmation because mutations are detected on complementary strands and so 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 individual 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. For the current mutagenized Arabidopsis populations that we are using, we find a density of 1 mutation per 235 kb, or approximately 4 point mutations per 8-fold pool gel (representing 768 plants; Greene et al., 2003).

A key advantage of high-throughput TILLING over competing methods is that the approximate position of each detected mutation is inferred from the size of the fragment, which greatly facilitates subsequent sequencing. Furthermore, the double-end labeling strategy provides confirmation within the pool screen, and further confirmation comes from identifying the same fragments in tracking down individuals. Therefore, sequencing is done with near certainty that a mutation exists within a small interval. Examination of a sequencing gel trace in the predicted location suffices to identify the mutated base and the substitution, and we use Sequencher trace analysis software (Gene Codes, Ann Arbor, MI) to facilitate this step. We have identified >3,000 Arabidopsis mutations in this way, typically using the readout from only the strand in which the primer is closer to the detected mutation. By contrast, methods that do not provide an approximate location for a detected mutation, such as DHPLC, require that the full amplified segment be interrogated by sequencing, and for a 1-kb segment this would require multiple runs to be carefully scrutinized. Detection of heterozygotes under such circumstances can be challenging, especially when peak heights vary, and false positives will greatly exacerbate this problem.

ARE BACKGROUND MUTATIONS A SERIOUS CONCERN?

The high densities of EMS mutagenesis that we aim for raise concerns about background mutations being

mistaken for mutations in target genes during phenotypic analysis. However, EMS-generated mutations at densities comparable to those in TILLING lines continue to be a basic learning tool for genetics, where background mutations obviously have not been a problem. On the one hand, mutations in genes expected to impact a phenotypic trait controlled by many genes, such as plant height or size or leaf shape, may be subject to epistatic interactions, and outcrossing to the wild type may be necessary. On the other hand, mutations in genes expected to impact a phenotype that is controlled by few genes are unlikely to produce phenotypes perturbed by background mutations, and outcrossing is not a prerequisite for analysis (Henikoff and Comai, 2003). Here's why: Following EMS treatment of seed, M1 plants are grown, selfed, and M2 individuals are propagated via single seed descent. M2 tissue is collected and DNA is extracted. Seeds are produced from M2 plants after a round of selfing; thus, each mutation will be in a ratio of one wild type to two heterozygotes to one homozygote in the resulting seeds. These seeds can simply be planted and genotyped because the two rounds of recombination and independent assortment prior to sowing the seeds scrambles the genome relative to the target site. Because only one-fourth of the seeds should be homozygous, by genotyping for the target locus lesion in a dozen or so plants and looking for a perfect correlation between genotype and recessive phenotype, the probability of being misled turns out to be vanishingly small. In other words, the two crosses that were carried out following mutagenesis unlink target and background mutations, so that it becomes highly unlikely for both a target and a background lesion to be homozygous in the same plants and only in those plants.

Based on mutation densities that we have measured in TILLING Arabidopsis and considering overall recombination rates, we have estimated that the probability of a closely linked lesion to be mistaken for one in the target gene is only approximately 0.0005. Furthermore, crossing members of the allelic series will bring together two independently mutagenized genomes, and so by typing and looking for a correlation between the heteroallelic pair and the recessive phenotype, a researcher can further reduce concerns about background mutations being mistaken for mutations in the target gene.

In conclusion, many phenotypes can usually be scored unequivocally in M3 populations. In certain cases, outcrossing might be necessary, but it should be possible to score most phenotypes after one or at most two generations. These strategies are the same employed in forward genetic screens for the past three-quarters century.

THE ARABIDOPSIS TILLING PROJECT: A HIGH-THROUGHPUT SERVICE

The high-throughput potential of TILLING led to the establishment of a TILLING facility in Seattle for

the Arabidopsis community at large, the Arabidopsis TILLING Project (ATP; Till et al., 2003). Consider the options available to a scientist who wants to elucidate the function of a sequenced Arabidopsis gene. She would probably first visit an insertional database such as the Salk Institute's T-DNA Express (Alonso et al., 2003). Assuming that our scientist finds her gene to be tagged and examines the resulting phenotype, three outcomes are possible: no mutant phenotype, a viable mutant phenotype, or a lethal phenotype. Probably in the second and certainly in the last case, our scientist would look for alternative tools to provide partial loss-of-function mutations in the gene. She may consider exploring the phenotype caused by any tagging element inserted in the 3' region of the gene (that may truncate the encoded polypeptide) or in the promoter, if available. Tags in the 3' region that reduce gene function would be very useful in the analysis. However, it is unlikely that they will provide an allelic series. Tags in the promoter would be difficult to interpret as they might affect expression of the gene in unpredictable ways. Our scientist may then explore the use of RNAi suppression (Waterhouse et al., 1998). However, RNAi suppression is laborious because it requires vector construction, transformation, and transgenic analysis. In addition, its outcome is unpredictable and often variable (Chuang and Meyerowitz, 2000; Jackson et al., 2003).

The scientist pursuing the function of this gene would find it advantageous to use TILLING. A search for mutations would be initiated, yielding approximately 10 mutations typically delivered 2 to 3 months later. Among these, our scientist 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.

In a significant minority of cases, there will be no available T-DNA insertion in the gene of interest. In such cases, TILLING could be employed to find knockout alleles, i.e. truncations. Ten TILLING mutations have an approximately 40% probability of including at least one truncation and 25 mutations have an approximately 70% probability, estimates that have been confirmed by analysis of the TILLED mutation set (Greene et al., 2003).

For a user, TILLING begins with a visit to the ATP Web site (<http://tilling.fhcrc.org:9366>), where she follows instructions for the interactive Web-based program CODDLE (for Codons Optimized to Detect Deleterious Lesions, <http://www.proweb.org/coddle>). CODDLE assists in all steps from selecting the gene region to ordering, after which TILLING begins. When mutations are discovered, confirmed, and sequenced, the user is automatically notified and sent to a Web page for coding and restriction site analyses and stock information. The series is also sent to The Arabidopsis Information Resource (TAIR; <http://Arabidopsis.org>) and formatted for entry into

their polymorphism/mutation database. In this way, information on each ATP mutation is conveniently accessible to anyone using TAIR's polymorphism/mutation entry tool, which provides links to map and sequence viewers, to ABRC seed stocks, and to ATP. Seeds for TILLING lines are ordered from ABRC using direct links from the TAIR entry. Thus, all TILLING work is performed on M2 populations by ATP, and all growth and analysis of M3 lines are performed by the user.

At its current capacity, ATP operates six or seven LI-COR analyzers in (typically) two daily shifts, and the team discovers an average of approximately 40 mutations per day. A user fee of \$500 for either the initial screen or for screening the remainder of the collection partially offsets ATP expenses. Nevertheless, most of ATP expenses are currently defrayed by a grant from the NSF Arabidopsis 2010 Project. Incremental technical advances and improvements in efficiency have gradually reduced the cost of TILLING since ATP was established, and by mid-late 2005, it is anticipated that user fees will cover all ATP operating costs. In the first 2 years of operation, ATP delivered approximately 250 allelic series totaling >3,000 sequenced mutations.

TILLING INFORMATICS

Several computer programs have been developed or adapted to facilitate the TILLING process. As described above, CODDLE provides the front end for TILLING (Till et al., 2003). It has multiple entry options for submitting a genomic sequence and for obtaining an exon-intron model for the gene of interest using public sequence databanks. CODDLE also aligns conserved protein regions from the Blocks database (<http://blocks.fhcrc.org>) with the gene model. CODDLE uses the Primer3 algorithm and reports suitable primers for amplification of the chosen fragment. CODDLE enters the information automatically into the order form, whereupon a submitted order is checked by BLAST searching, the user is billed, and the primers are ordered to initiate the process.

CODDLE was developed by Nicholas Taylor and Elizabeth Greene as a general tool that can also be used for polymorphism analysis and for conveniently designing primers for any organism and any mutagen. Whether for TILLING or for polymorphism analysis, there is a need to assess the effect of missense mutations. We use protein sequence conservation as the basis for evaluating whether a missense mutation is likely to have an effect on the encoded protein. This can be quite effective; for example, the conservation-based SIFT program predicts with approximately 75% accuracy whether or not an amino acid change is damaging to a protein (Ng and Henikoff, 2003).

Upon completion of the TILLING process, a report is sent to the user. The PARSESNP (for Project Aligned Related Sequences and Evaluate SNPs; <http://www.proweb.org/parsesnp/>) program reports map

and sequence positions for each result entered in graphical, tabular, and sequence formats (Taylor and Greene, 2003). The PARSESNP table also provides links to the appropriate stock center for ordering and reports restriction site information that can be used for subsequent genotyping. In addition, the TILLING report provides a SIFT evaluation of missense mutations (Ng and Henikoff, 2003). PARSESNP provides additional links for further analysis of missense mutations, including mapping of the mutations with respect to conserved protein blocks, and displays the location of the change on available 3D structures (Henikoff et al., 2002).

CODDLE, PARSESNP, and SIFT are general Web-based tools for functional genomics that have been adapted for TILLING. In addition, the TILLING team has implemented a variety of specialized programs for operations, data analysis, billing purposes, and other logistic needs. Although these programs were developed for ATP, they are adapted for other organisms as the need arises.

TILLING WORKSHOPS

Dissemination of TILLING technology to benefit plant research has been a major goal of our NSF-funded project. The process is sufficiently complex, both technically and logistically, that we decided to hold two-day workshops so that potential TILLING providers in the academic community can observe the process at firsthand. Workshop attendees, in groups of three to five, observe all steps of the high-throughput TILLING process and obtain current protocols on a collaborative basis.

Since the inception of workshops in November 2001, they have become increasingly popular and are now held almost monthly. In 2 years, our TILLING laboratory has hosted a total of 58 researchers from 13 different countries representing 20 different organisms. Several workshop attendees have subsequently established TILLING facilities at their own institutions, including Edwin Cuppen (Hubrecht Institute), Erin Gilchrist (University of British Columbia), and Cliff Weil (Purdue University). Workshops are also attended by researchers who have developed similar facilities independently, such as Charles Dearolf (Massachusetts General Hospital) and Jillian Perry (Sainsbury Institute). We believe that the workshop program is mutually beneficial, eliciting feedback and generating further collaborations while exposing participants to the challenges of a TILLING production operation.

TILLING OTHER ORGANISMS

Facile and efficient TILLING depends on the availability of two resources: a well-mutagenized population and genomic information. Chemical mutagenesis

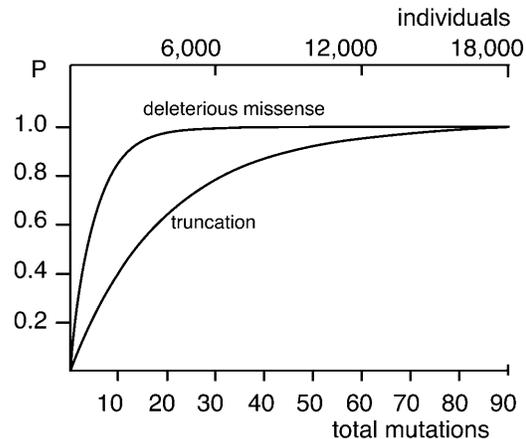


Figure 1. Probability of deleterious changes. The probability of finding either a truncation or a deleterious missense change is plotted as a function of number of mutations characterized. One-quarter of missense changes are assumed to be deleterious (<http://blocks.fhcrc.org/sift/SIFT.html>). Population sizes that are required to obtain these deleterious mutations are shown above, assuming a density of four mutations per megabase. This density has been found in the Arabidopsis population used by ATP (Greene et al., 2003); lower mutation rates will require screening proportionally more individuals.

is usually simple to carry out and exploit. Well-developed and tested protocols are available for organisms that are genetic models, such as Arabidopsis, maize, the worm (*Caenorhabditis elegans*), and the fruit fly (*Drosophila melanogaster*), and standard conditions for forward-genetics studies have been successful for TILLING. Notably, once a satisfactory mutation density has been achieved, the size of the mutant population sufficient for efficient TILLING is relatively small (<10,000; Fig. 1). There is limited information on mutagenesis dosage and mutation yield for crop plants. Anecdotal evidence suggests that the efficiency of mutagenesis varies from species to species, even within Arabidopsis (Henikoff and Comai, 2003). A better understanding of cellular factors affecting the success of mutagenesis should lead to broader application of this technology.

An important consideration is the structure of the mutagenized population library, which can vary considerably from organism to organism. For example, in Arabidopsis, after mutagenesis on M1 seed, we bank and TILL M2 DNA (the progeny of the M1) and bank and distribute M3 seed (the progeny of the M2). This is possible because an individual Arabidopsis plant produces thousands of seeds. However, in species that produce fewer than 100 seeds per individual, the M3 seed might be insufficient for distribution, and an additional generation would be necessary to produce and pool M4 seed from several M3 sibs.

Genomic information is useful but not absolutely necessary for TILLING. In theory, once primers have been demonstrated to amplify the target region of a gene, TILLING should be possible. In practice, knowledge of the genome sequence improves the

chance of success. For example, it allows in silico examination of mispriming and alternative targets. Polyploidy presents another challenge: If primers designed to amplify one locus in a tetraploid amplify the homeologous gene, pooling is changed as targets from two diploid genome equivalents are amplified per individual instead of one. Furthermore, the two targets might be amplified with different efficiency, further altering the pool composition. The problem can be addressed by determining the sequence of repeated loci and either designing locus-specific primers or adjusting the individual pooling scheme as needed. The considerable groundwork required for each target can delay high-throughput projects in unsequenced polyploid genomes.

TILLING PLANT GENES

Although there has been sufficient demand to keep ATP in continuous operation, Arabidopsis is rich in reverse-genetic resources, and TILLING is expected to be in greater demand where other methods are less applicable. Fortunately, the methodologies that we have developed and the pipeline that we have established for ATP are directly applicable to other organisms, and we and others have extended TILLING to a variety of organisms, especially crop plants. For example, Anawah has several programs for nontransgenic crop development, including for fruits and vegetables, cereals, soy, and peanuts (<http://www.anawah.com/programs/>).

The publicly funded ATP project has expanded to organisms other than Arabidopsis, becoming the Seattle TILLING Project (STP). STP collaborates with workshop attendees who are motivated to establish TILLING but are not prepared to make the substantial investment that is required. Once a mutagenized population is available, a pilot screen is performed, primarily to determine the suitability of a population for TILLING. Variations in mutation rate between organisms, between mutagens, and even between batches of seed or pollen are sufficient to necessitate pilot-scale screening before investing a major effort. Pilot screens also provide for an evaluation of DNA quality and other variables that affect the efficiency of TILLING. As part of our NSF Plant Genome Research Project (PGRP) award, we are able to offer TILLING pilot screening to parties who have potentially suitable mutagenized populations in organisms of interest to PGRP. We find that it is worthwhile to involve STP in the planning of a pilot screen at an early stage, when we can make recommendations based on our experiences with a variety of different organisms.

Several pilot projects have been performed in collaboration with workshop attendees who have established mutagenized populations. A pilot project is usually accomplished by preparing, normalizing, and arraying several hundreds of DNA samples from individual plants, ordering primers using CODDLE, and screening in the standard way. Pilot projects have

been performed on various mutagenized populations of rice, maize, soybeans (*Glycine max*), and Chlamydomonas with NSF PGRP support.

TILLING ANIMAL GENES

Plants are well suited for TILLING because seeds can be stored for long periods of time, allowing screening to be performed on the same mutant population indefinitely. Animals are also suitable for TILLING if there is an efficient strategy for germ plasm recovery. In two instances, this has been accomplished by saving the live progeny of screened individuals. Dearolf and co-workers used DHPLC to screen EMS-mutagenized *Drosophila*, obtaining an allelic series for the *awd* gene (Bentley et al., 2000). Plasterk's group used capillary sequencing to screen DNAs from ethylnitrosourea-mutagenized zebrafish to obtain an allelic series for the Rag-1 gene (Wienholds et al., 2002). In both cases, screens were completed in time to save the desirable mutant lines before aging and allele segregation became problems.

The use of live progeny rather than germ plasm storage means that screening should be completed within a single generation, and both studies were limited to single genes. Recently, however, Cuppen and co-workers used a modification of the STP method to obtain allelic series from 16 zebrafish genes within a single generation (Wienholds et al., 2003b). These zebrafish allelic series proved to be useful for analysis of gene function (Hurlstone et al., 2003; Wienholds et al., 2003a). The same group has also succeeded in TILLING several rat targets (Smits et al., 2004). Successful TILLING of zebrafish and rat is especially notable because the exon/intron structure of most vertebrate genes is poorly suited for finding damaging point mutations. Whereas the short introns of typical plant genes allow fragments to be chosen that are rich in coding sequence, the short exons of vertebrate genes usually need to be screened individually. To deal with these challenges, Cuppen's group has introduced important changes to the high-throughput protocol (Wienholds et al., 2003b). They perform PCR in two steps, first amplifying individual DNAs, then diluting and pooling 4-fold and amplifying with nested primers. The first amplification yields the same approximate final concentration of product regardless of the original concentration of template, and so relatively crude DNA preparations can be used without careful normalization that is necessary when pooling prior to amplification. Following nested PCR, CEL I digestion and LI-COR gel screening is performed, and whenever a mutation is discovered, all four samples in the pool are sequenced. To deal with the large number of reactions and transfers that are required using this strategy, Cuppen's group has implemented robotics in a 384-well format, such that all enzymatic steps are performed without human intervention.

Other solutions to the germ plasm storage problem have been applied to animal TILLING. Bruce Draper,

Cecilia Moens, and colleagues at Anawah have TILLED ethylnitrosourea-treated zebrafish using frozen sperm for germ plasm recovery (Draper et al., 2004). Draper and Moens are currently continuing this work in collaboration with the STP, where minor modifications of protocols have been implemented to improve efficiency of TILLING large-intron genes. We have also initiated *Drosophila* TILLING in collaboration with Charles Zuker (University of California, San Diego) and Barbara Wakimoto (University of Washington). To create permanent lines containing EMS-mutagenized chromosomes that are not subject to segregation and loss of mutations, Zuker's group has used standard balancer chromosomes (Ashburner, 1990). *Drosophila*'s small intron size and the high mutation rate that can be achieved suggests to us that the Zuker lines can be used to establish a *Drosophila* TILLING service modeled on ATP.

ECOTILLING

In addition to allowing efficient detection 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. We call this method Ecotilling (Comai et al., 2004).

Each SNP is recorded by its approximate position within a few nucleotides. Thus, each haplotype can be archived based on its mobility (Fig. 2). Sequence data can be obtained with a relatively small incremental effort using aliquots of the same amplified DNA that is used for the mismatch-cleavage assay. The left or right sequencing primer for a single reaction is chosen by its proximity to the polymorphism. Sequencher software performs a multiple alignment and discovers the base change, which in each case confirmed the gel band.

Ecotilling can be performed more cheaply than full sequencing, the method currently used for most SNP discovery. We simply screen plates containing arrayed ecotypic DNA rather than pools of DNA from mutagenized plants. Because detection is on gels with nearly base pair resolution and background patterns are uniform across lanes, bands that are of identical size can be matched, thus discovering and genotyping SNPs in a single step. In this way, ultimate sequencing of the SNP is simple and efficient, made more so by the fact that the aliquots of the same PCR products used for screening can be subjected to DNA sequencing.

FUTURE PROSPECTS

The need for allelic series of mutations for functional studies is not likely to abate in the near future, and the

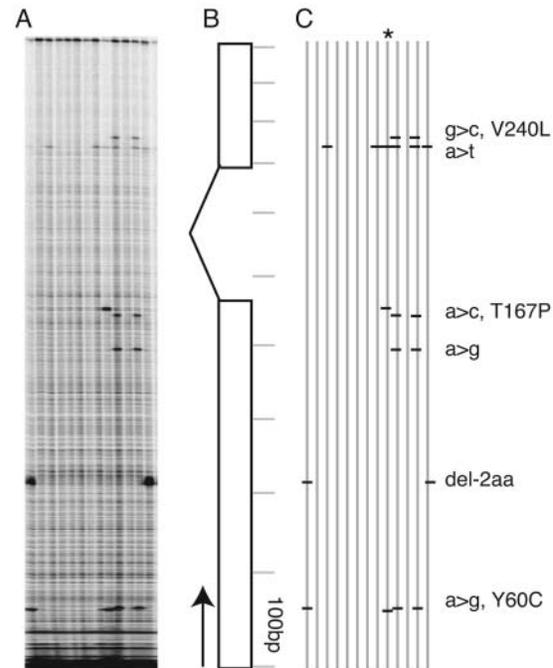


Figure 2. Detection of haplotypic diversity by Ecotilling. A, Part of an ecotilling image displaying the cleavage profile of 13 accessions at the *DRM1* locus. The DNA of each ecotype was mixed with that of the reference Columbia-0. After amplification with primers specific for the *DRM1* gene, the standard cleavage assay and LI-COR analysis was applied (Till et al., 2003). Only the IRD700 fluorescent channel is displayed. Polymorphisms are visible as bands above background, corresponding to the mobility of single-stranded cleavage products. B, Gene exon-intron model for the amplified portion of *DRM1*. The gene is oriented bottom to top to correspond to the LI-COR image. C, Five different haplotypes, including that of Columbia-0, are derived from the analysis in A. Sequence analysis confirmed the haplotypic classification, and its outcome is displayed on the right (Comai et al., 2004). *, Unsequenced haplotype.

increasing availability of genomic sequence will further fuel demand. Therefore, we expect that our high-throughput TILLING method, or something like it, will become increasingly popular, especially for agriculture, where there is so much useful knowledge to be gained from functional genomics and where non-transgenic methods are especially desirable.

Our ability to screen for point mutations on a production scale means that other steps in the process become limiting. Achieving high and consistent levels of mutagenesis while maintaining viability and fertility is a major challenge, especially for rice, where we continue to encounter difficulties in obtaining a suitably mutagenized population. Another challenge is what takes place after an allelic series is delivered: High-throughput TILLING discovers so many mutations that it sometimes can be a major effort for a user to adequately perform the necessary phenotypic analysis and genotyping.

TILLING depends upon the ability to detect mismatches in DNA heteroduplexes, but competition is

intense to develop other ways to discover and screen for single-nucleotide differences. For the long term at least, it is probably impossible to predict what technologies will prevail (Henikoff and Comai, 2003). For some tasks that TILLING technology is applied to, such as Ecotilling, brute-force sequencing might eventually prevail if costs plummet further and if the quality of heterozygote detection improves. Microarray technology looks very promising to us for the long term, but high cost and uncertain reliability are issues that still need to be addressed. The speed of mass spectrometry makes it a promising technology for the future. But for the near term, we think that heteroduplex analysis will continue to prevail. Whereas DHPLC and temperature gradient capillary electrophoresis are gaining in popularity for genotyping applications, we think that the advantages and low cost of mismatch cleavage with electrophoretic display makes it the technology to beat for mutation detection. We anticipate that incremental advances in TILLING methodology, together with improved software and logistics, will suffice for efficient reverse genetics over the next few years.

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LITERATURE CITED

- Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653–657
- Ashburner M (1990) *Drosophila*, A Laboratory Handbook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Bentley A, MacLennan B, Calvo J, Dearolf CR (2000) Targeted recovery of mutations in *Drosophila*. *Genetics* **156**: 1169–1173
- Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **97**: 4985–4990
- 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 Physiol* **126**: 480–484
- Comai L, Young K, Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson J, Burtner C, Oden AR, et al (2004) Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. *Plant J* **37**: 778–786
- Draper BW, McCallum CM, Stout JL, Slade AJ, Moens CB (2004) A high-throughput method for identifying ENU-induced point mutations in zebrafish. *Methods Cell Biol* (in press)
- 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
- Henikoff JG, Greene EA, Taylor N, Pietrokovski S, Henikoff S (2002) Using the Blocks database to recognize functional domains. In AD Baxevanis, D Davison, R Hogue, G Page, GD Stormo, L Stein, eds, *Current Protocols in Bioinformatics*. John Wiley & Sons, New York, NY
- Henikoff S, Comai L (2003) Single-nucleotide mutations for plant functional genomics. *Annu Rev Plant Physiol Plant Mol Biol* **54**: 375–401
- Hurlstone AF, Haramis AP, Wienholds E, Begthel H, Korving J, van Eeden FJ, Cuppen E, Zivkovic D, Plasterk RH, Clevers H (2003) The Wnt/beta-catenin pathway regulates cardiac valve formation. *Nature* **425**: 633–637
- Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* **21**: 635–637
- McCallum CM, Comai L, Greene EA, Henikoff S (2000) Targeted screening for induced mutations. *Nat Biotechnol* **18**: 455–457
- Middendorf LR, Bruce JC, Bruce RC, Eckles RD, Grone DL, Roemer SC, Sloniker GD, Steffens DL, Sutter SL, Brumbaugh JA (1992) Continuous, on-line DNA sequencing using a versatile infrared laser scanner/electrophoresis apparatus. *Electrophoresis* **13**: 487–494
- Nagy A, Perrimon N, Sandmeyer S, Plasterk R (2003) Tailoring the genome: the power of genetic approaches. *Nat Genet* **33** (suppl.): 276–284
- Ng PC, Henikoff S (2003) SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res* **31**: 3812–3814
- Oleykowski CA, Bronson Mullins CR, Godwin AK, Yeung AT (1998) Mutation detection using a novel plant endonuclease. *Nucleic Acids Res* **26**: 4597–4602
- Smits BMG, Mudde J, Plasterk RHA, Cuppen E (2004) Target-selected mutagenesis of the rat. *Genomics* **83**: 332–334
- Taylor N, Greene EA (2003) PARSESNP: A tool for the analysis of nucleotide polymorphisms. *Nucleic Acids Res* **31**: 3808–3811
- 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 Res* **13**: 524–530
- Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Natl Acad Sci USA* **95**: 13959–13964
- Wienholds E, Koudijs MJ, van Eeden FJ, Cuppen E, Plasterk RH (2003a) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat Genet* **35**: 217–218
- Wienholds E, Schulte-Merker S, Walderich B, Plasterk RH (2002) Target-selected inactivation of the zebrafish *rag1* gene. *Science* **297**: 99–102
- Wienholds E, van Eeden FJ, Kusters M, Mudde J, Plasterk RH, Cuppen E (2003b) Efficient target-selected mutagenesis in zebrafish. *Genome Res* **13**: 2700–2707