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Invited Review

Reverse-genetics by TILLING expands through the plant kingdom

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Abstract: There is a rich history of using induced mutations to deduce the *in vivo* function of genes and to develop crops with superior traits. The reverse-genetics strategy known as TILLING (Targeting Induced Local Lesions IN Genomes) was created to harness the power of induced mutations to target and recover lesions in specific genes. TILLING combines traditional mutagenesis strategies that have been in use since the early part of the 20th century with high-throughput discovery methods that are continuously being developed and improved upon. Because mutation induction and mutation discovery are not species-specific, TILLING is a general method that can be applied to most organisms. Since its first description in 2000, TILLING has been applied to over 20 plant species. We review here progress in the development and adaptation of TILLING for different plant species, describe the companion technology, Ecotilling, for discovery of natural nucleotide polymorphisms, and discuss future directions for reverse-genetics using induced mutations.

Key words: Mutation breeding, mutation discovery, mutation induction, reverse-genetics, TILLING

مقالة مرجعية

تنتشر في المملكة النباتيةTILLINGالوراثة العكسية بواسطة أمثلة

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مختبر علم الوراثة وتربية النبات ، القسم المشترك بين المنظمة الدولية للطاقة الذرية لاستخدام التقنيات النووية في الأغذية والزراعة ، والوكالة الدولية للطاقة الذرية ، مركز فيينا الدولي ، ص 100 ألف فيينا - 1400 ، النمسا

الملخص: هناك تاريخ عريض لاستخدام الطفرات المستحدثة لإستنتاج الوظيف العضوية للجينات و استنباط محاصيل ذات صفات لأفضل. إن استرتيجية الوراثة العكسية المعروفة باسم TILLING (استهداف تحللات موضعية في المادة الوراثية) تم وضعها لزيادة قوة الطفرات المستحدثة لاستهداف و استعادة القطع في الجينات. تتضمن TILLING استراتيجيات استحداث الطفرات التقليدية المستعملة منذ بدايات القرن العشرين و طرق الاكتشاف الفعالة التي يتم تطويرها و تحسينها بصفة مستمرة. ولأن اسحداث الطفرات واكتشافها ليس مرتبطاً بالنوع، فإن TILLING تعد طريقة عامة يمكن تطبيقها في معظم الكائنات الحية. ومنذ أول وصف لها في عام 2000، تم تطبيق TILLING في أكثر من عشرين نوع نباتي. نقدم هنا مراجعة للتقدم في مدى تطوير وأقلمة TILLING لانواع نباتية مختلفة، ونصف التقنيات المصاحبة، والبيئية لاكتشاف الصور المتشابهة الطبيعية للنيوكليتيدات، ونناقش الإتجاهات المستقبلية للوراثة العكسية باستخدام الطفرات المستحدثة.

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Introduction

Nucleotide polymorphisms are major components of heritable phenotypic variation and thus drivers of evolution and domestication. Where natural nucleotide differences are limited or difficult to recover, induced mutations can be created in the genomes of living organisms in order to generate novel genetic diversity (Muller, 1927; Stadler, 1928). Mutations have been a powerful tool for the discovery and characterization of major biological processes and gene functions. Their utility for practical applications may be best exemplified in plant breeding (Jain, 2010b; Jain et al., 2010). Currently over 3000 mutant crop varieties are registered in a database curated by the International Atomic Energy Agency (http://mvgs.iaea.org/). Traits improved via mutagenesis include enhanced yield and resistance to biotic and abiotic stresses (Jain, 2010b). The global economic impact of crops improved through mutation techniques is estimated in billions of US dollars annually (Ahloowalia et al., 2004).

The rapid increase in the acquisition of genomic sequence information in the past decade has enabled reverse-genetic approaches to directly probe the function of specific genes by testing the *in vivo* consequence of disruption or over expression of a gene on the phenotype of an organism (Tierney and Lamour, 2005). This is the "reverse" of traditional genetic analysis where phenotypes are observed and afterwards the gene causative for the phenotypic difference is cloned and validated. TILLING (Targeting Induced Local Lesions in Genomes) is a general reverse-genetics strategy that combines traditional mutagenesis with high-throughput mutation discovery methods (McCallum et al., 2000b). Treatment with mutagens can induce changes randomly in the genome of an organism, and so an optimal mutation density and population size can be achieved whereby multiple mutations can be recovered for every gene in the genome (Greene et al., 2003). Polymorphisms are typically discovered through PCR amplification of target gene regions of interest, followed by a mutation discovery step. A number of methods can be used to recover nucleotide variation, the most commonly applied being enzymatic mismatch cleavage followed by gel electrophoresis and band visualization (Till et al., 2004a).

A TILLING library consists of stored germplasm typically in the form of seed, and genomic DNA extracted from mutagenized material. Both DNA and germplasm can be stable for many years, allowing for screening for a variety of different traits and sharing of the library with a network of plant researchers as a public service (Till et al., 2003b). This represents an efficiency gain over traditional forward genetics or mutation breeding strategies where only a small subset of mutated alleles are recovered and maintained, while the majority of induced changes are discarded. Additionally, reverse-genetic strategies allow the selection of only potentially useful alleles for careful phenotypic evaluation. For example, screening a diploid population of 6000 chemically mutagenized Arabidopsis plants will yield < 30 potentially deleterious mutations (Greene et al., 2003; Till et al., 2003b). Thus the effort in phenotypic characterization and field evaluation can be reduced by two orders of magnitude when compared to forward genetics and mutation breeding. A disadvantage is that such estimations hold for monogenic traits where a single gene is controlling a phenotype, and mutation-based modification of polygenic traits can be more cumbersome. However, reverse-genetics strategies such as TILLING allow the recovery of potentially useful alleles that do not produce phenotypic differences alone, but do so when combined with others. Examples include genetic redundancy where disruption of multiple genes is required to reveal a phenotype, and in polyploid species where alteration of homeologous sequences is a requisite for the recovery of recessive trait (Enns et al., 2005; Slade et al., 2005). The probability of co-disruption of two or three target genes in a single plant, or the random combination of such alleles in cross breeding experiments is low, making TILLING an efficient way to recover phenotypes that are

either difficult or impossible by forward genetic approaches.

II. Mutagens and mutation densities

The basic protocol for TILLING begins with mutagenesis, followed by dissolution of chimeric tissues caused by mutagenic treatment, then isolation of DNA and storage of germplasm, followed by screening for induced mutation events (Figure 1, Till et al., 2006a; Till et al., 2006c). For plants, large mutant populations are generated by the treatment of seed, pollen or other tissues with a mutagen of choice. The most commonly used to date is seed mutagenesis using the chemical mutagen ethyl methane sulfonate (EMS, Table 1). EMS is considered advantageous because a high frequency of induced single nucleotide mutations distributed randomly throughout the genome can be achieved (Greene et al., 2003; Slade et al., 2005; Till et al., 2003b). Treatment with EMS causes primarily G:C to A:T transition point mutations, thus allowing a predictable recovery of a spectrum of alleles including knockouts and missense changes that can have varying effects on protein function. A different spectrum of mutations can be achieved using different mutagens. For example, treatment of rice seed with Az-MNU resulted in 67% G:C to A:T and 20% A:T to G:C point mutations (Till et al., 2007a; Till et al., 2007b). Less is known about treatment with physical mutagens. Fast-neutrons have been used to create reverse-genetic strategies exploiting induced large gene deletions, and treatment with gamma irradiation can result in a wider spectrum of mutations including small deletions that favour the recovery of knockout alleles (Li et al., 2002; Rogers et al., 2009; Sato et al., 2006). To date, the mutation densities estimated for various mutagenized populations are ranging between 1/23.3 kilobases and 1/6190 kilobases (Dong et al., 2009; Sato et al., 2006). In general, observed mutation densities for polyploid species tend to be higher than those in diploids (Table 1). This may be due to the protective nature that homeologous sequences provide against the loss of gene function due to the accumulation of deleterious alleles (Stadler, 1929). A notable exception to this trend was reported in the diploid *Brassica rapa* where a density of approximately 1 mutation per 60 kilobases was observed (Stephenson et al., 2010). This may be due in part to the paleopolyploid nature of the genome. As more TILLING populations are developed and screened a better picture of the effect of genome architecture on maximal mutation densities may emerge. A confounding factor, however, will be variable accuracies of mutation discovery assays performed in different laboratories.

Table 1. Examples of TILLING projects.

Organism	Common name	Mutagen	Mutation discovery method[a]	Population size	Mutation frequency	Reference
Arabidopsis thaliana	Arabidopsis	EMS	EMC	3000	1/200	(Greene et al., 2003; Till et al 2003)
Arabidopsis thaliana	Arabidopsis	EMS	EMC	3712	1/89	(Martin et al., 2009)
Avena sativa	Oat	EMS	MALDI-TOF MassCLEAVE; RAPD-PCR fingerprinting; DNA sequencing	2550	1/38; 1/20; 1/22.4	(Chawade et al., 2010)
Brassica oleracea	Broccoli, Crussels	EMS	EMC	960	1/447	(Himelblau et al., 2009)

	sprouts, Cauliflower					
Brassica rapa	Field mustard	EMS	EMC-ABI	6912	1/56	(Stephenson et al., 2010)
				2304	1/67	(Stephenson et al., 2010)
Cucumis melo	Melon	EMS	EMC	4023	1/573	(Dahmani-Mardas et al., 2010)
Hordeum vulgare	Barley	EMS	EMC	7348	1/500 (from 800 at 20mm to 140 at 50mm)	(Gottwald et al., 2009)
Hordeum vulgare	Barley	EMS	EMC-HPLC	4600	1/1000	(Caldwell et al., 2004)
Lotus japonicum	Lotus	EMS				(Perry et al., 2003)
Solanum lycopersicum	Tomato	EMS	EMC	3924	1/574	(Minoia et al., 2010)
				1297	1/322	(Minoia et al., 2010)
Solanum lycopersicum	Tomato	EMS	CSCE and HRM	8225 (M2) and 7030 (M3)	1/737	(Gady et al., 2009)
Triticum aestivum	Hexaploid wheat	EMS	EMCA	2348	1/23.3 to 1/37.5kb	(Dong et al., 2009)
Triticum durum	Tetraploid wheat	EMS	EMCP	1368	1/51	(Uauy et al., 2009)
Triticum aestivum	Hexaploid wheat	EMS	EMCP	1536	1/38	(Uauy et al., 2009)
Triticum durum	Tetraploid wheat	EMS	EMC	768	1/40	(Slade et al., 2005)
Triticum aestivum	Hexaploid wheat	EMS	EMC	1152	1/24	(Slade et al., 2005)
Oryza sativa ssp japonica	Rice	EMS	EMC	768	1/294	(Till et al., 2007b)
		Az-MNU		768	1/265	(Till et al., 2007b)
Oryza sativa ssp japonica	Rice	gamma ray	EMCA	2130	1/6190	(Sato et al., 2006)

Oryza sativa ssp japonica	Rice	MNU	EMC-cap	767	1/135	(Suzuki et al., 2008)
Glycine max	Soybean	EMS	EMC	529	1/140	(Cooper et al., 2008)
		EMS		768	1/140	(Cooper et al., 2008)
		EMS		768	1/250	(Cooper et al., 2008)
		MNU		768	1/550	(Cooper et al., 2008)
Pisum sativum	Pea	EMS	EMC	4704	1/200	(Dalmais et al., 2008)
Pisum sativum	Pea	EMS	EMC	3072	1/669	(Triques et al., 2007)
Sorgum bicolor	Sorghum	EMS	EMC	768	1/526	(Xin et al., 2008)
Zea mays	Corn	EMS	EMC	750	1/500	(Till et al., 2004b)

a EMC = enzymatic mismatch cleavage and fluorescence detection with LiCOR; EMCA= enzymatic mismatch cleavage with agarose gels; EMCP = enzymatic mismatch cleavage with non-denaturing polyacrylamide gel electrophoresis stained with EtBr; EMC-HPLC = enzymatic mismatch cleavage and denaturing HPLC; EMC-ABI = enzymatic mismatch cleavage and capillary gel electrophoresis; MALDI-TOF MassCLEAVE = Matrix-assisted laser desorption/ionization-time-of-light mass spectrometer; CSCE and HRM

III. Methods for mutation discovery

Successful TILLING depends not only on the mutation frequency which is driven by the correct selection of mutagen and its concentration/dose, but also the population size and the method used to identify specific mutations in the population. To date, the majority of employed mutation discovery technologies exploit physical differences in heteroduplexed DNAs that are created through hybridization of PCR products harbouring polymorphisms. Such heteroduplexed DNA molecules lack hydrogen bonding at the site of the SNP or indel. The first report on TILLING used denaturing HPLC as a mutation discovery method (McCallum et al., 2000a). This was soon replaced with a higher throughput method using enzymatic mismatch cleavage and fluorescence detection of cleaved bands fractionated by denaturing polyacrylamide gel electrophoresis (Colbert et al., 2001). Different single-strand specific nucleases and fragment analysis platforms have been further adopted (Cross et al., 2008). Other, non-enzymatic, techniques such as High Resolution Melt (HRM) and Confirmation Sensitive Capillary Electrophoresis (CSCE) have also been utilised for mutation screening for TILLING (Table 1). The ideal mutation discovery platform balances equipment and assay costs with sensitivity, throughput and accuracy. The correct balance will depend on the needs and the resources of a particular laboratory. A major drawback with many high throughput discovery methods is the reliance on costly specialised equipment and consumables. Efforts have been made to develop low-cost agarose and non-denaturing polyacrylamide gel platforms compatible with enzymatic mismatch cleavage using crude celery juice extracts, making TILLING feasible for most laboratories including those in developing countries (Dong et al., 2009; Raghavan et al., 2007; Sato et al., 2006; Uauy et al., 2009). However, thorough studies to compare sensitivity and accuracy of high-throughput versus lower cost and lower throughput assays have yet to be done. Issues such as polyploidy and the frequency of natural heterozygous polymorphisms can impact the effectiveness of different platforms (Chawade et al., 2010; Till et al., 2010). Care should be taken, therefore, when developing a mutation discovery platform to ensure that false discovery errors are minimized.

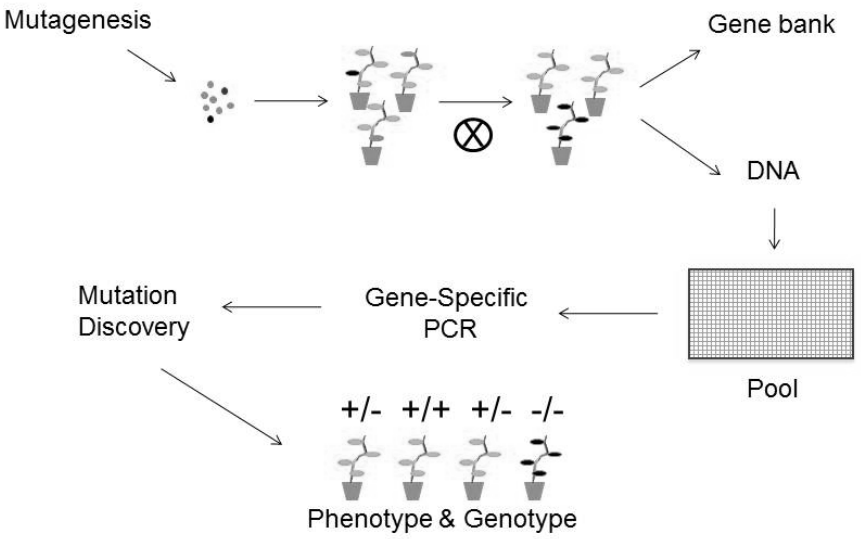

Figure 1. Traditional TILLING strategy for seed propagated crops.

Seed are treated with chemical or physical mutagens to induce random changes in the genome. Embryos are multicellular, and each cell accumulates different mutations. The resulting M1 plants are chimeric with different tissues harbouring different genotypes. Plants are self-fertilized and the resulting M2 plants are non-chimeric. A single-seed descent strategy is followed whereby tissue and seed from a single M2 plant is collected from the self-cross of the M1 for creation of the TILLING library. A DNA library of several thousand samples is typically prepared to ensure recovery of deleterious alleles. DNAs are typically arrayed in a 96 or 384 well format and samples pooled up to 8-fold prior to screening to increase the throughput of mutation discovery. Target genes are then amplified by gene-specific PCR and PCR products are screened for induced mutations. Recovered putative mutations are validated by sequencing and M3 seed from the TILLING gene bank harbouring desired mutations is selected for further evaluation and to test co-segregation of observed phenotypes with mutant genotypes. The same steps apply for vegetatively propagated species with the exception that the tissue remains in the M1 stage and chimeras are dissolved via mitotic propagation or avoided via mutagenesis of single cells.

IV. Ecotilling

The high-throughput and low cost methods for induced SNP and indel discovery developed for TILLING can also be applied for the discovery of naturally occurring nucleotide polymorphisms. This was discovered accidentally during efforts to develop the first large-scale TILLING service, the Arabidopsis TILLING Project. It was observed that a low percentage of plants of a different ecotype had contaminated the TILLING population through the discovery of multiple SNPs in single PCR amplicons. The method was developed using a core set of *Arabidopsis* ecotypes and thus named Ecotilling (Comai et al., 2004). The pilot work showed that Ecotilling provided a fast and accurate way to recover a wide range of haplotype diversity in target genes in natural populations. This has been exploited for wide range of applications including population diversity studies, methods to target rare potentially disease causing mutations in humans and association studies to identify allelic variants controlling disease susceptibility in melon (Gilchrist et al., 2006; Nieto et al., 2007; Till et al., 2006b). While bench methodologies are similar for both TILLING and Ecotilling applications, differences arise in data analysis as the frequency of natural SNPs can be many orders of magnitude greater than rare induced mutations (Figure 2, Comai et al., 2004; Greene et al., 2003). To assist in analysis of Ecotilling data, specialized computer software was developed (Henikoff et al., 2000; Till et al., 2006b; Zerr and Henikoff, 2005). Work continues in the development and adaptation of

Ecotilling for a wide range of crops. For example, optimizations for the co-discovery of SNPs in homeologous sequences in mixed populations of diploids and polyploids have recently been described (Till et al., 2010).

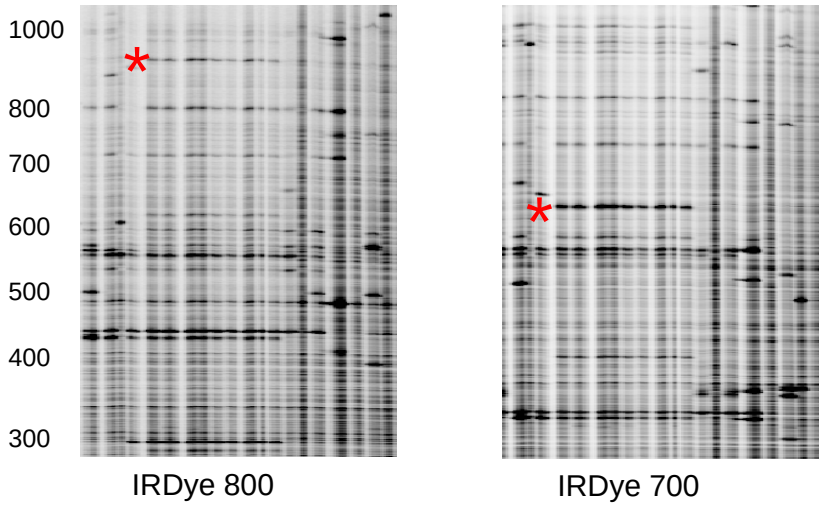

Figure 2. Traditional enzymatic mismatch cleavage and fluorescence detection used for discovery of natural polymorphisms in diploid and polyploid Musa accessions.

A ~1500 base pair gene target was PCR amplified with IRDye 700 (forward primer) and IRDye 800 (reverse primer) labelled primers and then subjected to mutation discovery using crude celery juice extract, followed by denaturing polyacrylamide gel electrophoresis and fluorescence detection using a Li-Cor DNA analyser. Gel image is cropped to show the range between 300 and 1000 base pairs in 20 of 96 assayed accessions. Cleavage products appear in both fluorescent channels, with the molecular weights of corresponding fragments summing to the size of the full-length PCR product (an example is marked with an asterisk). Careful primer design allowed accurate recovery of SNPs in homeologous sequences. [Adapted from (Till et al., 2010)].

V. Future perspectives

A major power of the TILLING strategy is that it relies on standard mutagenesis and mutation discovery methods that can be applied to most organisms. One limitation is the requirement for sequence information for the development of gene-specific primers used in many mutation discovery methods. While success can be achieved with limited sequence such as ESTs, the task is simpler and potentially more successful with more complete genomic sequence information (Till et al., 2003a). With novel next generation sequencing technologies under continual development, the expectation is that DNA sequencing costs will drop dramatically while throughput rapidly increases. This should enable rapid acquisition of *de novo* sequence and provide a new platform for mutation discovery for TILLING (http://tilling.ucdavis.edu/index.php/TILLING-by-Sequencing; Gilchrist and Haughn, 2010). It is therefore likely that the rate limiting and most challenging step will remain the development and maintenance of suitably mutagenized populations.

To date, mutagenized populations have been developed exclusively for seed propagated crops using either seed or pollen mutagenesis strategies (McCallum et al., 2000a; Till et al., 2004b). This is advantageous due to the ease of mutagenesis and long-term storage of seed. However, there is a large group of crop plants which are being maintained and propagated primarily or exclusively through the vegetative organs. These include staple foods for approximately 1 billion people in the developing world (http://faostat.fao.org/site/339/default.aspx). Efforts are being made, therefore, to establish efficient TILLING

protocols for vegetatively propagated species. This involves the development of *in vitro*, and cell suspension cultures, and methods for efficient induction, maintenance and recovery of mutations (Jain, 2010a; Jain et al., 2011). Efficient TILLING platforms in vegetatively propagated banana and cassava are currently in development. Preliminary results suggest that a high density of mutations can be induced and maintained through years of *in vitro* propagation (the authors of this review, unpublished). Identification of genes and their *in vivo* functions is of high importance in the context of increasing pressures on global food production. It is hoped that through continual development and transfer of modern technologies, sustainable intensification of crop production for food security can be realized.

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