Chapter 13
TILLING for Mutations in Model Plants and Crops

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Abstract A growing world population, changing climate and limiting fossil fuels will provide new pressures on human production of food, medicine, fuels and feed stock in the twenty-first century. Enhanced crop production promises to ameliorate these pressures. Crops can be bred for increased yields of calories, starch, nutrients, natural medicinal compounds, and other important products. Enhanced resistance to biotic and abiotic stresses can be introduced, toxins removed, and industrial qualities such as fibre strength and biofuel per mass can be increased. Induced and natural mutations provide a powerful method for the generation of heritable enhanced traits. While mainly exploited in forward, phenotype driven, approaches, the rapid accumulation of plant genomic sequence information and hypotheses regarding gene function allows the use of mutations in reverse genetic approaches to identify lesions in specific target genes. Such gene-driven approaches promise to speed up the process of creating novel phenotypes, and can enable the generation of phenotypes unobtainable by traditional forward methods. TILLING (Targeting Induced Local Lesions IN Genome) is a high-throughput and low cost reverse genetic method for the discovery of induced mutations. The method has been modified for the identification of natural nucleotide polymorphisms, a process called Ecotilling. The methods are general and have been applied to many species, including a variety of different crops. In this chapter the current status of the TILLING and Ecotilling methods and provide an overview of progress in applying these methods to different plant species, with a focus on work related to food production for developing nations.

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13.1 Introduction

Agricultural sustainability can be defined as a state where increases in food production are at least proportional to the rate of population growth. The projected world population by the year 2050 will be 9.2 billion; the bulk of which will be from developing and least developed countries. It is estimated that increases in population will be 4.3%, 61.2% and 156.4% for developed, developing and least developing nations, respectively (United Nations 2007). This suggests that major pressures for crop production will befall nations with the least resources available for crop improvement. Furthermore, many developing nations rely on so-called understudied crops where only minor investments have been made in plant biotechnology. The ability of developed nations to provide food assistance to meet the expected shortfall is in doubt due to increased demands on crops for non-food purposes such as biofuels (von Braun and Pachauri 2006).

Crop productivity is mainly affected by a variety of abiotic and biotic stresses. Approximately 70% of potential yield is lost due these stresses (Gale 2002). The major abiotic stresses that affect food production are drought, salinity and acidity. From the total global arable area, a third is affected by salinity and 40% by acidity (Gale 2002). Biotic factors such as diseases, insects and weeds also contribute to decreased yields. Crop production is increased either by expanding the arable area or using inputs such as improved seed, irrigation and chemicals. According to the Food and Agriculture Organization (FAO), about 80% of future increases in crop production in developing countries are predicted to come from agricultural intensification (FAO 2002). Based on this goal, crop breeders focus towards achieving improved cultivars that produce higher yields and at the same time tolerate to the sub-optimal soil and climatic conditions. By utilizing various breeding techniques, a number of improved cultivars from different species have reached farming communities and contributed to increases in global food production. The work on induced mutation alone led to over 2,000 officially registered crops from 1940 to 2000, of which 85% were the result of gamma- and x-ray mutagenesis (Maluszynski et al. 2000). Induced mutations have had a large impact in transforming the agriculture of the world, particularly in generating crop species having desirable traits (Ahloowalia et al. 2004).

Examples of improved traits with high impact are those which alter the architecture of the plant. Architectural changes include alteration in branching pattern and reduction in plant height. The major achievement of the Green Revolution in 1960s and 1970s was due to the introduction of semi-dwarf cultivars of wheat and rice along with crop production packages such as controlled use of irrigation, fertilizer, herbicide and fungicide. Semi-dwarf varieties have been produced by exploiting natural nucleotide variation and through induced mutations, and have led to tremendous increases in productivity. This led to an annual yield increment of about 80 kg/ha in wheat and rice produced in developing countries between 1975 and 1984 (Conway and Toenniessen 1999). According to the International Food Policy Research Institute, the Green Revolution represented the successful adaptation and transfer of scientific revolution in agriculture (IFPRI 2002). Currently, a number of genes affecting plant height have been identified from major cereal crops including wheat, rice and maize (for review, Wang and Li 2006).

With expanding genomic DNA sequence information from many plant species and increasing knowledge regarding the functional roles of specific genes in traits of agronomic importance, it is now possible to consider creating specific plant traits in a directed manner. One approach is to use transgenes to transfer a single or multiple genes of interest within or across species. Using this approach, scientists have been able to create rice producing provitamin A in the grain (Ye et al. 2000). While very powerful, transgenic approaches have been met with a high level of public disapproval and the use of the methods for food production is currently banned in many countries.

This calls for an alternative, non-transgenic, targeted approach for crop improvement in order to meet the increasing demand in food production. TILLING (Targeting Induced Local Lesions IN Genomes) is a general reverse genetic technique that uses traditional mutagenesis followed by high-throughput mutation discovery to identify deleterious lesions in specific target genes. The technique has proven to be robust and easily applied to a variety of different species including *Arabidopsis*, (Mccallum et al. 2000b; Colbert et al. 2001; Greene et al. 2003; TILL et al. 2003), rice (Sato et al. 2006; Till et al. 2007; Suzuki et al. 2008), maize (Till et al. 2004a), wheat (Slade et al. 2005), barley (Caldwell et al. 2004), soybean (Cooper et al. 2008), pea (Triques et al. 2007), and a number of non-plant species such as *Caenorhabditis elegans*, *Drosophila* and zebrafish (Gilchrist et al. 2006a; Winkler et al. 2005; Wienholds et al. 2003). We describe in this chapter the current status of TILLING in plants and discuss the potential role of TILLING in improving under-studied crops of the developing world. We also describe a related technique known as Ecotilling that is used to investigate natural nucleotide variations in the genes of interest.

13.2 TILLING Method

The TILLING strategy utilizes traditional mutagenesis followed by high throughput mutation discovery (Mccallum et al. 2000b; Colbert et al. 2001). The main steps in TILLING are mutagenesis, the development of a non-chimeric population, preparation of a germplasm stock, DNA extraction and sample pooling, screening the population for induced mutations, and the validation and evaluation of mutants (Fig. 13.1). The methods required for each step can be applied to many species, making the TILLING process broadly applicable. Mutants discovered by TILLING can be used for gene-function studies and can be introduced into breeding programs.

13.2.1 Selecting a Mutagen for TILLING

The choice and dose of mutagen for TILLING will dictate the spectrum and density of mutations to be found in individual plants. The best mutagen can be defined as one that produces the desired density and spectrum of alleles with the least pleiotropic

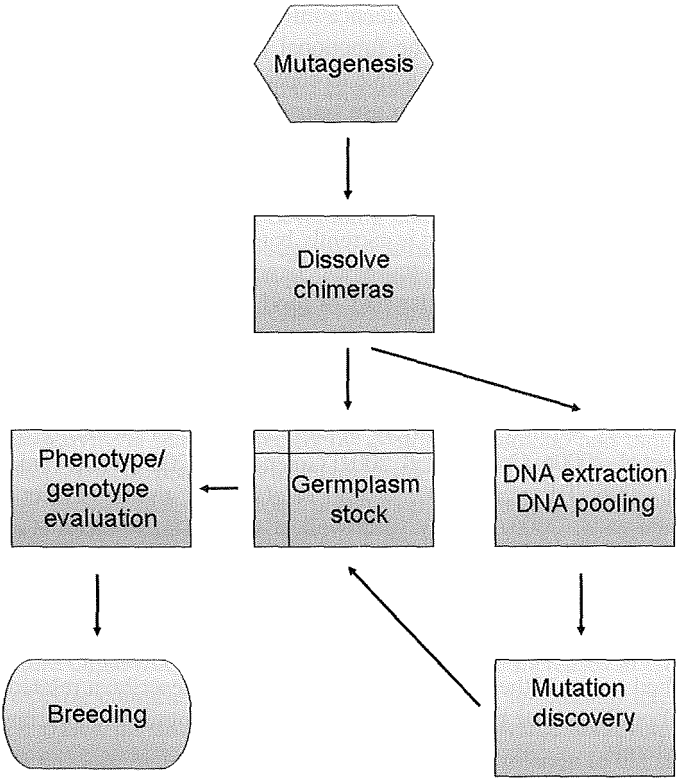

Fig. 13.1 A TILLING pipeline for gene function analysis and developing new crop varieties. A mutagenized population is prepared using a mutagen that primarily causes small lesions (single nucleotide polymorphisms, or insertions/deletions) randomly throughout the genome. Many mutagenic treatments produce a chimeric plant in the first generation. Chimeras are dissolved and a structured population is typically developed. A germplasm stock is prepared for long term storage of mutant lines, and DNA is extracted from each individual mutant. DNAs are pooled and the library of samples is screened for induced mutations in selected regions of target genes. Candidate mutants are removed from the germplasm stock and further characterized genotypically and phenotypically. Individuals or lines exhibiting the desired characteristics can be incorporated into a breeding program

consequences, low material and labor costs, and minimal health risks. Chemical mutagens have been used for many TILLING projects, with ethyl methanesulfonate (EMS) being most common (Table 13.1). Chemical mutagens are attractive for TILLING because protocols for their use in forward genetic screens have previously been developed for many species, the mutagens are readily available, easy to use, and produce predictable heritable nucleotide changes at high densities. Many chemical mutagens, such as EMS, primarily produce single nucleotide changes in the genome (Greene et al. 2003; TILL et al. 2003). Single nucleotide changes are useful because truncation and missense changes are obtained, providing a broad allelic series. Nonsense mutations that create premature stop codons, and splice site

Table 13.1 Spectrum and density of mutations reported in TILLING populations

Organism	Mutagen	Tissue mutag -enized	Mutation discovery method	Population screened	Targets screened	Induced mutation	Spectrum of mutation	Density of mutation (mutations/kb)	Reference
Arabidopsis	EMS[a]	Seed	Mismatch cleavage and fluorescence detection	3000	192	1890	>99% GC–>AT	~1/200	Greene et al. (2003), TILL et al. (2003)
Barley	EMS	Seed	Mismatch cleavage and denaturing HPLC	4600	2	10	70% GC–>AT	~ 1/1000	Caldwell et al. (2004)
Maize	EMS	Pollen	Mismatch cleavage and fluorescence detection	750	11	17	>99% GC–>AT	~1/500	Till et al. (2004a)
Pea	EMS	Seed	Mismatch cleavage and fluorescence detection	3072	5	60	100% GC–>AT	1/669	Triques et al. (2007)
Rice	EMS	Seed	Mismatch cleavage and fluorescence detection	768	10 10	27 30	70% GC–>AT 11% AT–≥GC	~1/290	Till et al. (2007)
	Az-MNU[b]						67% GC–>AT 20% AT–>GC	~1/270	
Rice	MNU[c]	Seed	Mismatch cleavage and capillary detection	767	4	24	92% GC–>AT	1/135	Suzuki et al. (2008)
Rice	Gamma ray	Seed	Mismatch cleavage and agarose gel detection	2130	25	6	50% Transversion 17% transition–33% small indel[d]	1/6190 kb	Sato et al. (2006)
Soybean	MNU	Seed	Mismatch cleavage and fluorescence detection	529	7	32	~90% GC–>AT	1/140	Cooper et al. (2008)
	EMS			768	7	12	~90% GC–>AT	1/140	
	EMS			768	7	25	~90% GC–>AT	1/250	
	EMS			768	7	47	~75% GC–>AT[e]	1/550	
Wheat	EMS	Seed	Mismatch cleavage and fluorescence detection	1920	7	>200	~99% GC–>AT	1/40 (tetraploid) 1/24 (hexaploid)	Slade et al. (2005)

[a] Ems: ethyl methanesulfonate.
[b] Az-mnu: sodium azide methyl nitrosourea.
[c] Mnu: methyl nitrosourea.
[d] Exact nucleotide changes not reported.
[e] No significant difference in spectrum between this population and other soybean populations.

mutations that inhibit proper intron splicing can knock out gene function providing null alleles. Missense changes can produce variable affects on protein function and are valuable for obtaining phenotypes from essential genes that would cause organism death if completely knocked out. In addition, missense mutations can provide a broad range of phenotypes that can enhance gene function studies, and be useful in breeding. The utility of point mutations in breeding may be best exemplified by the value of the missense mutations causing the semi-dwarf sd1 phenotype in rice that fuelled the Green Revolution (Sasaki et al. 2002).

The spectrum and density of induced mutations identified in TILLING screens varies by mutagen and by species (Table 13.1). In *Arabidopsis*, maize, and wheat, EMS produced > 99% GC:AT changes that could be identified by TILLING (Greene et al. 2003; TILL et al. 2003; Slade et al. 2005). A study of ~2000 EMS induced mutations in *Arabidopsis* showed that mutations were essentially induced randomly throughout the genome (Greene et al. 2003). Random mutagenesis means that mutations can be discovered anywhere in the genome regardless of target size provided the proper balance of population size and mutation density is obtained. With a high density of ~1 mutation per 200 kb in *Arabidopsis*, a relatively small population of 3,000 mutant individuals is typically screened to deliver ~10 alleles per ~1.5 kb target region (http://tilling.fhcrc.org/arab/status.html). Approximately 5% of EMS induced mutations are truncation changes, and 48% missense changes (Greene et al. 2003). A high mutation density reduces the population size needed to deliver the desired number of alleles, and thus reduces the cost of sample preparation and screening. Mutation density is, therefore, a key factor in determining the scale and costs of a TILLING project. For gene function studies and publicly available TILLING services, a strategy has been to achieve a high mutation density to reduce mutation screening costs. A lower mutation density may be desirable for breeding applications.

Other mutagens can be considered for TILLING. For example, mutagenesis of rice seed with sodium azide plus MNU (methyl-nitrosourea), and soybean seed with MNU produced populations with mutation spectra and densities similar to that observed with EMS (Table 13.1). Inducing mutations with physical mutagens has a long history beginning with the work of Muller exposing Drosophila to X-rays (Muller 1928). With Muller's initial report, scientists embarked upon an extensive use of radiation to induce mutations and 30 years later were reporting extensively on induced mutagenesis mediated by ionizing radiation (Bauer 1957; Mac-Key 1956; Singleton 1955; Smith 1958; Konzak 1957; Sparrow 1956; Gaul 1958; Miksche and Shapiro 1963; Hough and Weaver 1959). More recently, Maluszynski et al. (2000), and Ahloowalia et al. (2004) have provided very comprehensive reviews on officially released induced crop mutants, most of which had been induced to mutate using physical agents.

While induced mutations caused by physical mutagens have a long history in both basic and applied research, less is known about the spectrum and density of lesions in crops caused by such treatments. One of the most widely used physical agents is gamma irradiation. A population of rice mutagenized with gamma-irradiation was recently used in TILLING screens, yielding only point mutations and small

deletions (Sato et al. 2006). The density of induced mutations in this population, however, was very low (1 mutation per 6,190 kb). One explanation for the low density is that gamma-irradiation produced larger genomic lesions in this population that went undetected because of the method used to find mutations. Because large lesions would be, on average, more deleterious to gene function than SNPs (Single Nucleotide Polymorphisms), fewer such lesions will be tolerated in a genome. If a mutagenic treatment induces a mixture of large and small lesions, the result would be a lower maximal density of lesions than, for example, what could be obtained with a mutagen causing only SNPs. Studies of gamma and carbon irradiation of *Arabidopsis* pollen showed that small and large deletions and inversions can occur due to mutagenic treatment (Naito et al. 2005). Together, this suggests that gamma-irradiation of plant material can cause a combination of large and small lesions, and that obtainable mutation densities may be lower when compared with chemical mutagenesis. More work is to be done to characterize the spectrum and density of gamma-induced lesions in plant genomes, and the effects of using different doses of mutagen.

Large genomic lesions such as deletions are useful for creating phenotypes, especially when knockouts are desired, including the deletion of tandemly repeated genes, such as those involved in disease response. For example, fast neutron mutagenesis was used to create deletions in *Arabidopsis* and rice that could be detected by a simple PCR based assay (Li et al. 2001). When compared to mutagens causing primarily SNPs, fast neutron mutagenesis is disadvantageous because mutation densities are much lower, and a ~>tenfold larger population is required to ensure the recovery of a deletion in any gene in the genome. Furthermore, the mutations will cause primarily knockouts versus the allelic spectrum of knock-out and missense changes caused by point mutations. However, physical mutagens that create large deletions and inversions may produce a higher frequency of dominant alleles. This may be desirable, particularly when studying polyploid species or vegetatively propagated crops. An additional advantage of physical mutagenesis is that the procedure is easily centralized and plant material is non-toxic and safe to ship after treatment. For example, the FAO/IAEA joint programme provides a gamma-irradiation service that is free to its member states (http://www.iaea.org/OurWork/ST/NA/NAAL/agri/pbu/agriPBUmain.php). When creating mutagenized populations for TILLING, it may be worthwhile considering several different mutagens for a combined strategy for the recovery of both large deletions and SNP mutations. The method of mutation discovery, however, is an important factor in choosing a mutagenesis strategy (see Section 13.2.3).

13.2.2 Selecting Tissue for Mutagenesis

The optimal tissue for large-scale mutagenesis for TILLING can be defined as one that produces non-chimeric plants harbouring a high density of induced mutations and requires the lowest inputs of labor and propagation time. Because most point

mutations causing phenotypes will be recessive, an additional criterion is that mutations can be easily made homozygous. The optimal tissue may therefore change from species to species and depending on the resources and needs of the scientist. Seed mutagenesis has been the choice for many plant species (Table 13.1). For chemical mutagenesis, seeds are soaked in a mutagen for a set period of time, washed to remove the mutagen, and then sown. The first generation, called M_1, is genotypically chimeric, with different parts of the plant having different genotypes because of the multicellular nature of the embryo at the time of mutagenesis (Henikoff and Comai 2003). To obtain heritable mutations, the plant must be propagated to produce a generation that is non-chimeric before DNA can be collected for TILLING screens. When possible, self-fertilization is used. Populations are typically structured using a single seed descent strategy such that one M_2 progeny from the self cross of the M_1 is selected to create a line for TILLING (for example, Till et al. 2003; Caldwell et al. 2004). Tissue is collected from this M_2 individual for later DNA extraction and mutation screening. M_3 seed are collected from a self pollination of the M_2, and this becomes the germplasm stock. Mutations identified in the M_2 sample are either heterozygous or homozygous. If M_2 plants selected for the TILLING population are chosen randomly, a Mendelian ratio of 2:1 heterozygous to homozygous mutations should be observed in the TILLING screen (Greene et al. 2003).

Non-structured, or bulk, populations can also be used for TILLING. As with a structured population, the first non-chimeric generation can be screened. A disadvantage of the bulk strategy is that siblings sharing the same mutations will be screened, increasing the time and cost of mutation discovery. The strategy may therefore be best for projects focusing on specific phenotypes that can easily be identified and sorted to make a phenotype enriched population. The utility of screening a phenotype-enriched population was shown for *Lotus japonicus*. Perry and colleagues created a mutagenized population of *Lotus* that was enriched for nodule and root-specific phenotypes. TILLING screens of 288 plants led to the identification of 6 novel alleles (Perry et al. 2003). The advantage of this strategy is that the size of the enriched population is much smaller than the entire population, saving time and money on mutation screening. The enriched population will be depleted in mutations causing non-enriched phenotypes, and the complete mutagenized population is screened when targeting genes hypothesized to cause other phenotypes.

Efficiencies in the TILLING pipeline can be gained by choosing tissues for mutagenesis that create a non-chimeric individual in the M_1 generation. For maize, pollen has been soaked with EMS and then applied to ears of unmutagenized tester strains (Till et al. 2004a). Each kernel on the resulting ear contains a unique collection of heterozygous mutations. A single kernel defines a line and DNA for mutation screening can be extracted from the M_1 generation.

In vitro methodologies also have great potential for rapidly achieving homozygosity and minimising, if not totally nullifying, the need for the dissociation of chimeras in mutagenic populations. Cell suspension cultures, relying on totipotency of cells, involves the production of cell lines from callus followed by the

regeneration of plantlets through somatic embryogenesis. Typically, this involves the culturing of single cells and small cell aggregates that proliferate and complete a growth cycle while suspended in liquid medium. Since Nickell's (1956) pioneering work with cell suspension cultures of *Phaseolus vulgaris*, reproducible protocols have been produced for other plant species. This ability to culture individual plant cells, from which whole plantlets will arise, therefore permits the treatment of individual cells with mutagens. The plantlets that arise from each treated cell are genetically similar leading to significant gains in time, especially as the need for several cycles of regenerations required to dissociate chimeras are eliminated. Because plants from culture develop via mitosis, induced mutations will remain heterozygous in adult tissues. To uncover recessive phenotypes, mutations must first be made homozygous.

Totipotency is also exploited in the regeneration of doubled haploids (DHs), when the chromosome number of gametic cells, i.e. pollen and egg cells, is doubled prior to regeneration of a plant (Forster et al. 2007). This process could be incorporated into induced mutagenesis by the treatment of these gametic cells prior to regeneration of the doubled haploids. With spontaneous and/or induced doubling of the haploid chromosomes, homozygous individuals are produced, providing the most rapid mechanism for attaining homozygousity with the greatest fidelity (Szarejko and Forster 2007). By facilitating the possibility of targeting either the haploid or doubled haploid cells for mutation treatment, the authors (Szarejko and Forster 2007) posit that a mutation is captured in a homozygous, pure line. These individuals are homozygous for all loci including the mutated segments of the genome being targeted for detection. The savings in time and cost are significant as recessive mutations usually are not detectable until the M_2 or later generations of sexual propagation through self-fertilization. Rapid technological advances have resulted in the availability of reproducible DH protocols for over 250 plant species (Maluszynski et al. 2003) covering most plant genera. Enthusiastic incorporation of DH in induced mutagenesis should therefore be successful and lead to significantly enhanced efficiency in delivery processes. A major disadvantage, however is that unlike self fertilization, where individual progeny are expected to be homozygous for ~25% of alleles, a plant created by DH procedures will be homozygous for all mutations, including deleterious background mutations that can confound phenotypic analysis. Because of this, DH plants will tolerate a lower mutational load, thus mutation densities by this method will be lower, necessitating larger populations to discover sufficient alleles. For vegetatively propagated crops that produce viable pollen, DH may be the only method for creating homozygous alleles.

Where protocols for somatic embryogenesis, through cell lines or friable embryogenic calli, for instance, are not available, plantlets could also be regenerated but at relatively lowered levels of homozygousity and enhanced levels of chimeral sectors through in vitro nodal segments. For obligate vegetatively propagated crops for which microspore cultures followed by chromosome doubling are impracticable, the in vitro nodal segments strategy, while not optimal, is more efficient than using stem cuttings or other vegetative propagules itemised by Micke and Donini (1993) which include tubers, dormant buds; rhizomes; dormant shoots; etc.

If this route is taken, induced crop mutants would be developed, even if genetic constitution, including other mutation events, are unknown (Van Harten 1998) but due consideration must be given to planning strategies for efficient dissociation of chimeras. Where multicellular meristematic tissues have been used as starting materials for the induction of mutations, several cycles of regenerations are required to dissociate chimeras in order to approximate the homohistont state. The optimal number of regenerations to remove chimerism may vary by species, propagation techniques and the type of chimeras (mericlinal, periclinal or sectorial chimeras), and should be determined empirically. For instance, Novak et al. (1990) and Roux (2004) suggested two different numbers of regenerative cycles, 6 and 4, i.e. M_1V_6 and M_1V_4, respectively as the minimum for banana. Also, because mutations are expected to be heterozygous, choosing a mutagenesis treatment that increases the frequency of dominant alleles should be a top priority.

13.2.3 DNA Extraction, Pooling and Mutation Discovery

The DNA extraction method used for TILLING should produce a sufficient yield and quality of genomic DNA that is stable for the duration of the project. These parameters are determined empirically (Till et al. 2006b). Genomic DNA samples are typically prepared from single plants. Samples are normalized to a common concentration and pooled together to reduce the cost of mutation screening. Pooling of up to eight samples has been used for TILLING (for example, Colbert et al. 2001; Till et al. 2004a; Cooper et al. 2008). Several strategies for pooling samples have been used. In a one dimensional pooling strategy, each plant is represented in a single pool of eight, and 768 unique samples (96 pools) can be screened in a single 96 well TILLING assay. Pools with putative mutations are then deconstructed and the eight samples comprising the pool are screened individually (Colbert et al. 2001; Till et al. 2006b). A two-dimensional pooling strategy has also been used (Till et al. 2007; Cooper et al. 2008). In this strategy, individual samples are arrayed in 8 × 8 grids and pools are made by combining samples by columns and by rows (Fig. 13.2). An individual sample is therefore represented in two unique pools in the assay, and a positive mutation produces a signal in two assay lanes. The repetition of samples in the two dimensional strategy means that at a constant sample pooling (i.e. eightfold), only half the number of samples are screened per assay when compared to the one dimensional method (384 versus 768 for 96 well assays). The advantage of the two dimensional strategy is that mutations are validated and individuals identified in a single step, streamlining the process and potentially minimizing false positive and false negative error rates (Till et al. 2006a).

A variety of methods are available for the discovery of unknown nucleotide polymorphisms. For example, traditional Sanger sequencing, denaturing HPLC and enzymatic mismatch cleavage have all been used for mutation discovery in reverse genetic screens of populations treated with chemical mutagens (McCallum et al.

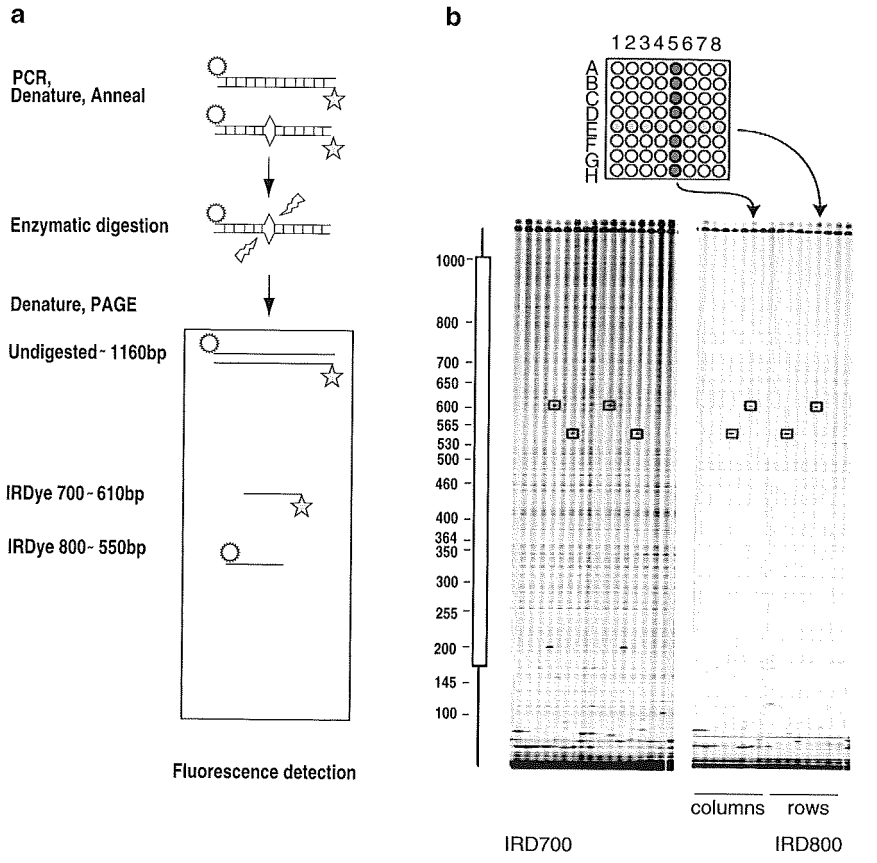

Fig. 13.2 Enzymatic mismatch cleavage and fluorescence detection for mutation discovery in TILLING. Schematic diagram of enzymatic mismatch cleavage (panel A). PCR is performed with primers end-labelled with IRDye700 (*circle*) and IRDye800 (*star*) fluorescent dyes. After PCR, amplicons are denatured and annealed to form heteroduplexed molecules. Mismatched regions are cleaved by treatment with a single-strand specific nuclease, typically an extract containing CEL I. Products are then denatured and size fractionated by denaturing polyacrylamide gel electrophoresis. DNA is visualized by fluorescence detection, typically with a LI-COR DNA analyzer. The sum of the molecular weights of the cleaved products equals the molecular weight of the undigested full length product. Using two different dyes allows the approximate position of the mutation on the PCR amplicon to be estimated. A two dimensional pooling strategy was used to discover chemically induced mutations in rice (panel B). Sixty-four samples are arrayed in an eight by eight grid (*top*). Column pools are prepared by combining all samples in a common column (numbered 1–8). The same is done for row pools (A-H). Samples from column and row pools from a grid are loaded on adjacent lanes of the gel (*bottom*). A positive signal found in a column pool will replicate in a row pool. The lanes provide coordinates for determining the identity of the mutant sample in the grid (E5 in the marked example). Panel B modified from Till et al. 2007

2000a; Colbert et al. 2001; Wienholds et al. 2002; Caldwell et al. 2004). Factors important in choosing a mutation discovery platform include equipment cost, maintenance costs, reagent costs, and reliability and error rates of the assay. Currently,

the most common platform is enzymatic mismatch cleavage using single-strand specific nucleases followed by fluorescence detection (Fig. 13.2, Table 13.1). PCR is performed using a forward primer 5′ end labelled with the IRDye700 dye, and a reverse primer labelled with IRDye800 to produce amplicons of approximately 1.5 kb (Fig. 13.2a). Products are then denatured and annealed to form heteroduplexes between polymorphic amplicons. Heteroduplexes are cleaved with a single-strand specific nuclease and products are size fractionated by denaturing polyacrylamide gel electrophoresis (PAGE). Fluorescent signals are detected using the Li-Cor DNA analyzer (Fig. 13.2b). The molecular weights of the cleaved fragments indicate the position of the nucleotide change within ~10 bp (Greene et al. 2003). The exact nucleotide change is then determined by sequencing. Mung bean nuclease, S1 nuclease, CEL I nuclease, and other members of the S1 family have been shown to work in the TILLING assays (Till et al. 2004b; Triques et al. 2007). The accuracy of using a crude extract of CEL I to detect nucleotide polymorphisms in pools was determined using human SNPs. In a blind study testing discovery efficiencies using previously identified human polymorphisms, a false discovery rate of 2% and false negative error rate of 7% was reported for screening samples in eightfold pools that were arrayed two dimensionally (Till et al. 2006a). The high throughput and low cost nature of this mutation discovery method for TILLING is exemplified by fees charged by TILLING services that operate on a full-cost recovery basis... At the time of writing, users pay USD dollar 2,000 for an allelic series of ~12 induced mutations from ~3,000 mutagenized *Arabidopsis* lines (http://tilling.fhcrc.org/files/user_fees.html). The sensitivity of the mutation discovery method in a production setting is shown by the recovery of the expected 2:1 Mendelian ratio of heterozygous to homozygous mutations in the samples from M_2 *Arabidopsis* plants (Greene et al. 2003). If mutation discovery sensitivity was limiting one would expect to find less heterozygous mutations because they are represented at half the concentration of homozygous polymorphisms in pooled samples.

The TILLING method has been further streamlined through the development of computational tools for assay design and data analysis. The CODDLe (Codons Optimized to Detect Deleterious Lesions) program uses the expected spectrum of mutations for a given mutagen to calculate the ~1.5 kb region of a gene that contains the highest number of potential mutations that could adversely affect gene function (McCallum et al. 2000b, http://www.proweb.org/coddle). Because a high density of mutations can be achieved, sufficient alleles are typically obtained by choosing the best 1.5 kb region of the gene rather than screening the entire gene in overlapping 1.5 kb segments. In addition to counting nonsense and splice site changes, CODDLe uses protein homology to predict the severity of missense changes. The CODDLe output links directly to Primer3 primer design software (Rozen and Skaletsky 2000). Approximately 90% of primers ordered for the *Arabidopsis* TILLING Project service ordered using this system have produced sufficient amplification product for TILLING (http://tilling.fhcrc.org/arab/status.html). Once mutations have been discovered by TILLING, they can be graphically displayed with the PARSESNP program that incorporates the SIFT program to predict if missense mutations are likely to affect protein function (Taylor and

Greene 2003; Ng and Henikoff 2003; http://www.proweb.org/parsesnp). Analysis of Li-Cor gel data for TILLING and Ecotilling is aided by the PC/Mac program Gelbuddy (Zerr and Henikoff 2005; www.gelbuddy.org). Gelbuddy provides molecular weight calibration, automated lane discovery, and automated band discovery. All programs described here are freely available and can be used for additional applications not linked to TILLING or Ecotilling.

Rapid advancements in next generation sequencing platforms hold great promise for increasing the efficiency of mutation discovery for TILLING. Next-generation sequencing can be defined as any number of new technologies that promise to dramatically increase the speed and reduce the cost of DNA sequencing when compared to traditional Sanger sequencing. Technologies include currently commercial pyrosequencing and mass spectroscopy based platforms to the developing field of nanopore sequencing (Hall 2007; Shendure et al. 2008). While whole genome sequencing is not at the time of writing cost competitive for the discovery of the approximately several hundred induced mutations in a highly mutagenized diploid plant in a population of thousands of plants, progress is being made in strategies for selective enrichment of desired targets that drive costs downward. Strategies include pre amplification of selected targets by PCR and genomic selection by microarray hybridization (for example, Albert et al. 2007).

Several large scale TILLING services including the Seattle TILLING Project, the Maize TILLING Project and the Rice TILLING Project, are currently evaluating next generation sequencing technologies for TILLING using the Solexa and ABI SOLiD platforms (Henikoff S, Comai L, personal communication, and http://genome.purdue.edu/maizetilling/). Next generation sequencing technologies can be used to discover all types of lesions, making them suitable for mutation discovery regardless of the choice of mutagen and type of lesion created. In addition, mutation discovery in polyploids may be more efficient using sequencing methodologies that collect data from a single starting molecule because it is not necessary to target specific homeologues (see Section 13.4). The accuracies and costs reported for TILLING using enzymatic mismatch cleavage will provide valuable data as a baseline to evaluate next generation sequencing technologies for TILLING, and can provide direction for future technological advancements. In the near-term, implementing new sequencing technologies will remain expensive, and likely financially feasible only for large scale facilities. Indeed, the high cost of laboratory infrastructure and training for mutation discovery, along with the generality of the methods, suggests that centralized mutation discovery facilities will be the most efficient and cost effective means to support TILLING for the scientific community. However, it is expected that the cost of sequencing technologies will drop dramatically, and early successes by large scale facilities will be valuable for smaller groups interested in TILLING. While new technologies are very exciting, it is important to note that the current state of the art for TILLING mutation discovery, enzymatic mismatch cleavage followed by fluorescence detection, is not a bottleneck in terms of time or cost for the TILLING pipeline as outlined in Fig. 13.1. The major bottleneck lies in mutant characterization, and large efficiencies can be gained by developing rapid and low cost phenotyping procedures. Advances in mutation discovery will, however,

greatly benefit TILLING facilities that provide fee-based mutation discovery, but not phenotyping, services.

13.3 Examples of TILLING Projects

The TILLING method has continued to gain in popularity since its first description in 2000. There are many active TILLING projects; some are at the level of fully operational TILLING services, while others are just at the beginning of platform development for a new species. TILLING has been adapted to over 20 species, and many groups host web sites describing projects and progress (Table 13.2).

13.3.1 High-Throughput Services

TILLING projects can be grouped into two broad categories: internally focused projects aimed at addressing specific biological problems, and service-based projects aimed at providing screening services to one or more research community. The first publicly available TILLING service was the Arabidopsis TILLING Project run by the Seattle TILLING Project (http://tilling.fhcrc.org/). Since its inception in 2001, the Arabidopsis TILLING Project has delivered ~8,000 mutations to the research community (http://tilling.fhcrc.org/arab/status.html). With the success of Arabidopsis, the STP went on to develop TILLING in a variety of different organisms for both large and small scales. The STP initiated pilot projects in maize, rice and Drosophila have been developed into large-scale screening services (http://genome.purdue.edu/maizetilling/, http://tilling.fhcrc.org/fly/Welcome_to_Fly-TILL.html, and http://tilling.ucdavis.edu/index.php/Main_Page,)

Other groups have also developed TILLING services (Table 13.2). For example, the TILLMore facility at the University of Bologna in Italy offers fee for service screening of barley (*Hordeum vulgare* cv Morex). The Scottish Crops Research Institute (SCRI) also offers screening services for EMS induced mutations in barley. The Lotus TILLING facility at John Innes Centre in UK provides screening services for EMS induced mutations in *Lotus japonicus*. The Plant Genomics Research Unit, URGV in France currently offers screening services in tomato (TOMATILL), pea (PETILL), and Rapeseed (RAPTIL) (http://urgv.evry.inra.fr/UTILLdb). An internationally organized Grain Legumes Technology Transfer Platform (GL-TTP) runs a Medicago TILLING platform. GABI-TILL is another large consortium with 13 collaborating institutions in Germany that offer screening services for collaborators. The consortium focuses on crops such as barley, sugar beet and potato. The number of services highlights the demand for induced mutations and reverse genetic screening services in a large number of organisms. Based on this, we expect the number of TILLING services to continue to grow.

Table 13.2 Examples of global TILLING projects in plants

TILLING platform/ project	Host organization	Organisms[a]	URL of the project or platform
Arcadia Biosciences TILLING	Arcadia Biosciences, USA	Wheat, castor, other crops	http://www.arcadiabio.com/toolbox.php
Barley TILLING	SCRI, Scotland	Barley	http://www.scri.ac.uk/research/genetics/BarleyTILLING
CAN-TILL	University of British Columbia, Canada	Arabidopsis, brassica, *C.elegans*	http://www.botany.ubc.ca/can-till/
GABI-TILL Project	GABI Consortia, Germany	*Arabidopsis*, barley, sugar beet, potato	http://www.gabi-till.de/main/main/home.html
Lotus TILLING	John Innes Centre, UK	Lotus	http://www.lotusjaponicus.org/tillingpages/homepage.htm
Maize TILLING Project	Purdue University, USA	Maize	http://genome.purdue.edu/maizetilling/
MBGP TILLING	Multinational Consortia	*Brasicca napus, B. oleracea, B. rapa*	http://www.jic.ac.uk/staff/lars-ostergaard/tilling/tilling.htm
Medicago TILLING platform	Grain Legumes Technology Platform, European Consortia	Medicago	http://www.gl-ttp.com/products_services/technical_services/genomic_resources_from_glip/functional_genomics/medicago_tilling_platform/
Peanut TILLING	Georgia Peanut Commission	Peanut	http://www.gapeanuts.com/growerinfo/research/research2006.asp
Rice TILLING platform	Nat. Inst. Of Genetics, Japan	Rice	http://www.nig.ac.jp/section/kurata/kurata-e.html
Seattle TILLING Project	Seattle, USA	*Arabidopsis*, Drosophila (services). Pilot projects in rice, soybean, and other organisms	http://tilling.fhcrc.org/
Soybean Mutation Project	Southern Illinois University, USA	Soybean	http://www.soybeantilling.org/
TILLmore	DiSTA, Bologna, Italy	Barley morex	http://www.agrsci.unibo.it/~salvi/tillmore/index.htm

(continued)

Table 13.2 (continued)

TILLING platform/ project	Host organization	Organisms[a]	URL of the project or platform
University of California-Davis TILLING Project	UC Davis, USA	Rice, tomato, wheat	http://tilling.ucdavis.edu/index.php/Main_Page
URGV TILLING Project	URGV, France	Pea, rapeseed, tomato	http://www.versailles.inra.fr/urgv/analysis-cropFunctionalGen.htm
USDA Bean TILLING Project	USDA	Beans	http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=197922
USDA Sorghum TILLING Project	USDA	Sorghum	http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=180731

[a]List of organisms may be incomplete.

13.3.2 Other TILLING Projects

Small scale pilot projects are useful to evaluate the efficacy of a TILLING strategy (choice and dose of mutagen, method for identifying mutation, etc.), and have led to a large number of original research articles (for example, Table 13.1). This indicates that TILLING is a powerful technique to investigate genetic alterations in organisms with different genome size and ploidy level. For example, the hexaploid wheat with genome size of 1.6×10^{10} bp (over 3.5 times more than rice) was successfully used in a TILLING project to create a variety producing low amounts of amylose (Slade et al. 2005).

In addition to public screening services, large-scale projects devoted to specific biological questions have been developed. For example, the Centre for Novel Agricultural Products (CNAP) at the University of York has recently initiated a project to obtain high yielding cultivars of *Artemisia annua*, the source of the anti-malarial component artemisinin, using the TILLING technique (CNAP 2006). Artemisinin-based therapies (ACTs) remain one of the most effective treatments against different species of *Plasmodium*, the causal agents of malaria (Enserink 2007). Because of its high demand, the drug is becoming more expensive and is not affordable in poorer countries where the epidemics of malaria are high. Thus TILLING could have a major impact in the production of medicines, exemplifying the utility of TILLING for crop improvement for non-food production. TILLING can also be envisioned as a tool for the production of superior crops for other non-food uses such as biofuel production.

13.4 Challenges for Crops

Success with organisms such as maize, rice, soybean and wheat show the utility of the TILLING method for crops. Some crops face unique challenges for TILLING. Polyploidy may at first seem an obstacle, but work with wheat shows that TILLING provides a targeted method for developing phenotypes that may not be obtainable when using forward genetic strategies (Slade et al. 2005). With TILLING in polyploids, severe alleles in different homeologues can be discovered independently, and then combined by cross-fertilization to provide the desired phenotype. The main technical challenge for TILLING in polyploids is the need to PCR amplify only one gene target (homeologue) per TILLING assay using enzymatic mismatch cleavage for mutation discovery. In theory, multiple gene targets could be amplified and screened at the same time, however in practice it has been observed that this approach leads to the failure to detect mutations (Cooper et al. 2008). The same issue arises when screening closely related genes in diploids. Amplification of a single gene target can be accomplished by careful primer design (Slade et al. 2005), or by removing the undesired gene target by restriction digestion of the genomic DNA prior to PCR (Cooper et al. 2008). Lack of genome sequence data can also be considered a major

challenge. While inefficient, primers for TILLING can be developed using cDNA or EST sequence data. PCR products from primers are then tested to determine intron location and size. This is the strategy used for pilot projects at the STP, and by the Maize TILLING Project (http://genome.purdue.edu/maizetilling/index.htm).

One concern in implementing TILLING in breeding will be the mutational load, or the number of background mutations in a particular plant. Background mutations can modify, mask, or enhance the desired phenotype, or can produce an unlinked and unwanted phenotype. Knowing the mutation density from TILLING assays allows an estimation of the total number of mutations in a single plant. This information can be used when deciding the best course of action to deal with background mutations for gene function studies and breeding. For example, using the mutation frequency for *Arabidopsis* mutants, it was estimated that through genotype segregation analysis of the progeny from a heterozygous mutation identified in the M_2 generation, a phenotype would be misattributed at a frequency of ~0.0005 (Henikoff and Comai 2003). Furthermore, using known recombination frequencies, the number of backcrosses required to remove the desired number of background mutations can be calculated. Because the majority of point mutations causing phenotypes are likely recessive, and background point mutations will be distributed randomly from plant to plant, a rapid strategy to remove the effects of background mutations is by crossing two strong alleles together as is done in a complementation or allelism test. This results in all background mutations becoming heterozygous. Because the probability that two plants randomly accumulated background mutations deleterious to the same gene is vanishingly small, the phenotypes resulting from the complementation cross can be attributed to the target gene with high confidence. This strategy is suggested by the *Arabidopsis* TILLING project for gene function analysis (http://tilling.fhcrc.org/files/FAQ.html).

For vegetatively propagated crops, the challenges of developing a suitable TILLING population mainly lie in the choice of mutagen, tissue to be mutagenized and the ability to either obtain a sufficient density of dominant mutations, or to create double haploid plants. Work with vegetatively propagated crops is just beginning and the information regarding efficient approaches will have to be gathered through trial and error. Where resources for in vitro techniques are limited, small TILLING populations can be generated and then rapidly screened, and only plantlets with interesting mutations maintained for further analysis. This live population strategy has been previously used for Drosophila and Zebrafish (Bentley et al. 2000; Winkler et al. 2005; Wienholds et al. 2003).

13.5 The Role of TILLING in Orphan Crops

Understudied crops also known as orphan, underutilized, lost, or disadvantaged crops, play major roles in the economy of developing countries (Naylor et al. 2004). They provide income for subsistence farmers and serve as staple food for largely low income consumers. Because of genetic diversity and localized selective

pressures not found in major crop monocultures, these under researched crops can perform better than major crops of the world under extreme soil and climatic conditions prevalent in developing world, particularly in Africa (Ketema 1997; Nelson et al. 2004; Williams and Haq 2002). The robust phenotypes and underlying genetic information will likely become valuable as crop performance is affected by climate change.

13.5.1 The Need to Improve Orphan Crops

As their name suggests, despite their huge importance, understudied crops have so far received little attention from the scientific community. Due to lack of genetic improvement, these crops produce inferior yield in terms of quality and quantity. Moreover, some of these crops produce a variety of toxins which are hazardous to humans if consumed before post-harvest processing (Getahun et al. 2003; Vetter 2000).

In general the major bottlenecks affecting the productivity of under-researched crops are genetic traits such as low yield (for example, in tef (*Eragrostis tef*), finger millet (*Eleusine coracana*)), poor in nutrition cassava (*Manihot esculenta*), enset (*Ensete ventricosum*)), and production of toxic substances (cassava and grass pea (*Lathyrus sativus*)). Environmental factors such as drought, soil acidity and salinity, pests, diseases and weeds also contribute for large loss in quality and quantity of yield. Hence, an agricultural revolution is needed to increase food production of understudied crops in order to feed the ever increasing population in the developing world.

13.5.2 TILLING Projects in Understudied Crops

The advancement of genomic techniques and information is making the process of moving an organism from "understudied" to "well-studied" easier than ever before. Genome sequencing projects are in progress or proposed for several understudied plant species (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj), and collections of cDNA and EST sequences continue to grow, enabling comparative genomics studies that can provide predictions regarding gene function. With sequence information and gene function predictions, TILLING becomes ideally suited for understudied species that lack many of the tools common to well studied species. Because it is a non-transgenic method, it can be easily incorporated into breeding programs without regulatory restrictions. In this section, we describe examples of progress in TILLING two understudied crops and describe unique challenges for these species.

Cassava (manioc; *Manihot esculenta* Crantz), a herbaceous plant with starchy tuberous storage roots and a major food security crop especially in sub-Saharan

Africa has an annual cultivation acreage of about 16 million hectares. The edible storage roots constitute the main source of dietary calories for about 500 million people in Africa, Asia and Latin America (http://www.ciat.cgiar.org/ciatinfocus/cassava.htm). The estimated production for 2007 was 200 million tons (http://www.fao.org/docrep/010/ah864e/ah864e06.htm#32). In addition to starch, the storage roots contain significant amounts of calcium, phosphorus and vitamin C (Nutrient Data Laboratory, USDA). The cassava plant is hardy, tolerant to drought and capable of producing far more meaningful yields under low input agriculture and on poor soils than most major crops. Cassava production is constrained by myriad problems, with the most significant being biotic stresses (principally bacterial and viral diseases). Additionally, the high contents of the anti-nutritional factor, cyanogenic glucosides (CG) a deadly poison, coupled with low protein contents make the crop unsuitable as a staple. Another bottleneck to large-scale cultivation of cassava is the rapid post-harvest physiological deterioration – the production of scopoletin as a wound response mechanism which in turn darkens the roots within 48 h of harvest (Ceballos et al. 2004; Vetter 2000). Being the cheapest source of starch, the crop has potentials for replacing other starch sources for varied industries including pharmaceutical, textile, paper and bioethanol applications.

The Joint FAO/IAEA Programme has therefore initiated a TILLING project in cassava as a means for directly querying the putative mutants for lesions in target genes before further field trialling. The mutants developed jointly with International Centre for Tropical Agriculture (CIAT) are an important resource for developing the cassava TILLING and Ecotilling platforms. The cassava genome is currently being sequenced and the imminent increase in sequence information will lead to a routine integration of TILLING in cassava induced mutagenesis as a crop improvement strategy. Conversely, induced mutagenesis and efficient reverse genetics strategies such as TILLING will contribute significantly to a rapid use of the bourgeoning sequence information in functional genomics studies in cassava.

Parallel to setting up the cassava TILLING platform, efforts are also being directed at mitigating the effects of chimerism in cassava mutants through the development of validated protocols for the integration of somatic embryogenesis in cassava mutagenesis. Protocols for profusely regenerating plantlets from friable embryogenic callus are available and current efforts target their adaptation to induced mutations (using both physical and chemical mutagens). Being able to eliminate chimeras, regenerating plantlets from a single mutagenised cell, will greatly enhance the predictive value of lesions that are identified through TILLING.

Tef (*Eragrostis tef* (Zucc.) Trotter) is grown annually on over 2.5 million hectares of land mainly in Ethiopia. The plant adapts to diverse climatic and soil conditions and grows better than other cereals both under drought and water-logged conditions (Ketema 1997). Unlike other cereals, the seeds of tef can be stored easily without losing viability under local storage conditions, since it is not attacked by storage pests (Ketema 1997). Tef is free of gluten hence safe for people with severe allergies to wheat gluten (NRC 1996; Spaenij-Dekking et al. 2005). Compared to other cereals, however, the average seed yield from tef is one of the lowest. Lodging is the major constraint to increasing the yield of tef (Ketema 1997).

The tef TILLING project based at the University of Bern in Switzerland is recently initiated with financial support from Syngenta Foundation for Sustainable Agriculture and University of Bern, and scientific collaboration with University of Georgia, FAO/IAEA Programme, and Ethiopian Institute of Agricultural Research. The main goal of the project is to obtain semi-dwarf tetraploid tef lines which are resistant to crop lodging. Since tef has a tall and tender stem, it is susceptible to damage by wind and rain. In addition, when the optimum amount of fertilizer is applied to increase the yield, a high incidence of lodging occurs. As a consequence, the yield from the crop is severely reduced in terms of total grain yield and quality. In general, the yield loss due to lodging is estimated to about 30% for tef. So far, the project has generated over 4,000 M_2 mutagenized lines to be utilized in TILLING.

The genes to be investigated are selected based on the information from other cereals. The dwarf plants of wheat that tremendously increased the yield during the Green Revolution in 1960s and 1970s contain the mutated Reduced height-1 (Rht-B1 and Rht-D1) gene (Peng et al. 1999). The commercially popular rice cultivar known as *semi-dwarf* (*sd-1*), is also defective in a gene involved in gibberellin biosynthesis (Spielmeyer et al. 2002). In addition, the maize *brachytic2* (*br2*) mutants and its ortholog in sorghum *dwarf3* (*dw3*) are also characterized by compact lower stalk internodes (Multani et al. 2003). The height reduction in these plants results from the loss of a P-glycoprotein (PGP) that modulates polar auxin transport in maize stalk (Multani et al. 2003). The Tef TILLING Project will identify from tef several of the orthologous genes indicated above and use the sequence information to screen the mutagenized population. The ability to create dwarf tef by TILLING should have a positive impact on yield when combined with optimized fertilizer use.

13.6 Ecotilling

The same methods that were developed for high throughput TILLING can be applied for the discovery of natural nucleotide polymorpshisms in populations. Proof of principle experiments were performed with 196 *Arabidopsis* accessions known as ecotypes, where the name Ecotilling derives. Screening revealed that multiple polymorphisms, including SNPs, indels (insertion and deletions) and variation in microsatellite repeat number could be efficiently discovered within a single amplicon, contrary to what might be expected from nuclease cleavage of end-labeled DNA (Comai et al. 2004). It was hypothesized that the ability to observe multiple cleaved fragments from end labelled substrates was due to incomplete digestion by CEL I on any one duplexed DNA molecule in the population of molecules being digested. A total of 55 distinct haplotypes were discovered in five ~1 kb *Arabidopsis* gene fragments. To unambiguously assign haplotypes to individual plants, samples were not pooled prior to screening. To uncover homozygous polymorphisms between samples, and equal concentration of DNA from the

sequenced Columbia accession was added to each sample. Screening therefore revealed unique polymorphisms between the test sample and the known sequence of the Columbia reference. Based on comparisons with data collected by Sanger sequencing, it was concluded that low false positive and false negative error rates were associated with Ecotilling.

The accuracy of the Ecotilling method was further explored in a blind study for human SNPs in a collection of samples previously characterized by resequencing (Till et al. 2006a). Five ~ 1.5 kb gene targets were selected for Ecotilling that overlapped regions in genes that were part of a Sanger-based resequencing SNP discovery effort by another group. Ninety samples were in common between the resequencing and Ecotilling data sets. A 4% false discovery and 2% false negative error negative rate was reported for screening unpooled samples. Samples pooled eightfold were screened to increase the efficiency of discovering rare SNPs that may be involved in human disease, and 2% false discovery and 7% false negative error rates were reported. Importantly, new rare alleles were discovered by Ecotilling that went unreported in the resequencing data, suggesting that the high sensitivity of Ecotilling makes it a useful strategy for discovering rare alleles such as those associated with cancer. Using estimates based on cost-recovery fees from large-scale TILLING services, it was estimated that a large scale effort to identify rare cancer mutations by Ecotilling would be ~50-fold less expensive than by using traditional Sanger sequencing.

One important difference between TILLING and Ecotilling is the amount of data that is produced. For a well mutagenzied diploid population, one can expect to find approximately four induced mutations when screening a 1.5 kb region in 768 individuals. The Ecotilling work in *Arabidopsis* and humans revealed hundreds of polymorphisms when screening 96 samples with a 1.5 kb gene target. With an informatics load increase approaching two orders of magnitude, the task of identifying and managing polymorphic bands in Ecotilling data can become a bottleneck. To aid in the analysis of Ecotilling gels, the GelBuddy program was developed for the analysis of Li-Cor gel images (Zerr and Henikoff 2005). The freely available program provides automated lane identification and molecular weight calibration, plus both manual and automated polymorphism band discovery (Till et al. 2006a, www.gelbuddy.org). Importantly, automated band detection discovered a few polymorphisms that were overlooked during manual analysis. While the overall accuracy was lower for automated band detection than by an expertly trained human, the method is objective and can be combined with manual editing to achieve low error rates and reduce data analysis labor.

As with TILLING, the use of the Ecotilling method continues to grow as it is applied to new organisms. Indeed, natural populations may be the best or only resource to study and exploit nucleotide diversity in species where mutagenesis is difficult or impossible. For example, Ecotilling was used to characterize nucleotide variation in 41 accessions of western black cottonwood (*Populus trichocarpa*), to estimate linkage disequilbrium, heterozygosity and nucleotide diversity (Gilchrist et al. 2006b). At the FAO/IAEA joint programme, Ecotilling is being applied to accessions of banana, cassava, and rice with the hopes that alleles important for

biotic and abiotic stress response can be identified. This work is progressing as mutagenized populations for TILLING are being developed, and so data gathered in Ecotilling can be used when choosing targets to screen for induced mutations (Till, BJ, Jankowicz-Cieslak, J, Nakitandwe, J, Bado, S, Afza, R, and Mba C, unpublished). Nieto and colleagues used Ecotilling to screen for candidate polymorphisms in melon associated with resistance to Melon necrotic spot virus (Nieto et al. 2007). A modification of the Ecotilling method using CEL I double strand cutting and an agarose gel readout platform was used to characterize salmon (Garvin and Gharrett 2007). The ability to incorporate Ecotilling into a production screening pipeline is exemplified by the Maize Tilling Project, that now includes screening 48 maize accessions with standard TILLING screens (http://genome.purdue.edu/maizetilling/EcoTILLING.htm). The characterization and exploitation of natural nucleotide diversity will undoubtedly play an important role in crop improvement in the twenty-first century.

13.7 Conclusions

In less than a decade, TILLING has moved from a proof of concept to a well accepted reverse genetic method that has been applied to over 20 different species. Large-scale TILLING services have delivered thousands of induced mutations to the international research community. Ecotilling has also grown in popularity. With increasing pressures on crop productivity expected in the twenty-first century, we predict the use of induced and natural mutation to elucidate gene function and to enhance phenotypes will continue to gain in importance. TILLING and Ecotilling can provide enhanced efficiencies to breeding pipelines and will therefore be useful in meeting expected demands. The advancement of new mutation discovery techniques should provide further improvements to the TILLING and Ecotilling processes.

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