‘TILLING’ – A Reverse Genetics Tool For Crop Improvement

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Abstract

Mutations are playing a crucial role in the evolutionary process. The spontaneous or induced variability can be used in functional genomics as well as for crop improvement. Various attempts were made in past to improve the crop by induced variability using physical and chemical mutagens. The vast information and sequence data bases accumulated during last a few years facilitated the gene directed modifications and gene discovery using reverse genetics tools. TILLING (Targeting Induced Local Lesions IN Genome) is a reverse genetics approach which make high through put screening of the single nucleotide variations and allows the direct identification of beneficial nucleotide and amino acid changes in genes with known functions and their use as the genetic markers for selection. Induced mutagenesis or accessions containing natural polymorphisms (Eco-TILLING) combined with TILLING provides a powerful tool for non-transgenic method for crop improvement as it works within the genome of the plant itself and thereby, is free of the regulatory hurdles imposed on genetically modified organisms. The range of alleles that can be developed via TILLING in a short time is unparalleled and unlikely to be found elsewhere in the pool of germplasm accessible to plant breeders.

Key words: Mutagenesis, EMS, SNP Discovery, TILLING, Non-transgenic.

Introduction

Crop improvement has a long history as key agronomic traits have been selected over thousands of years during the domestication of crops. More recently, this progress has been accelerated as the green revolution has brought about great increases in crop yields (Khush, 2001). With the advent of genomics in the last 25 years, opportunities for crop improvement have continued to grow and may help to meet future challenges of food production and land sustainability. The food demand curve is rising sharply and it is clear that to feed the future population, improving traits in major crops especially such as yield, disease resistance, drought tolerance, nitrogen use efficiency, enhanced nutritional quality etc. are major priority research areas. There is a need to develop new high yielding varieties by a combination of traditional plant breeding and new tools of biotechnology. With the advent of crop genomic projects worldwide, information about new genes that could be used for improving crops is becoming available which should be utilized to devise new strategies, such as TILLING (Targeting Induced Local Lesions IN Genome) for the benefit of the agriculture research especially as GM route will provide limited solutions to the major problems faced by the agriculture sector. Although transgene-based (GMOs) biotechnology approach has been implemented for the genetic modification of crops, the process is very laborious in most species. Moreover, time to market is long due to regulatory hurdles. Additionally, when applicable, it cannot keep up with the speed at which candidate genes of agronomical importance are identified and there is a need to test their function in the crops. Mutation breeding offers an alternative way to manipulate endogenous genes for the improvement of crops without transgenics.

Spontaneous mutation, the naturally occurring heritable changes to genetic material which played a pivotal role in biological evolution and formed the basis for speciation and domestication of both crops and animals, can be artificially induced and supports the maintenance of biodiversity. There have been more than 2700 officially released mutant varieties from 170 different plant species in more than 60 countries (FAO, 2008). This not only increase biodiversity but also provide breeding material for conventional plant breeding, thus directly contributing to the conservation and use plant genetic resource. The genetics behind mutation breeding includes differences in the sensitivity of different genotypes and plant tissues to different mutagens. Mutation induction continues to contribute to crop improvement, using physical mutagens such as gamma ray, X-ray, fast neutron, and chemical mutagens such as EMS (ethyl methane sulphonate) and sodium azides. Recently, new physical mutagens, such as ion beam radiation and cosmic rays, have been proven to be effective for inducing mutations.

Screening DNA sequences for mutations and polymorphisms has become one of the most challenging, often expensive and time-consuming obstacles in many molecular genetic applications, including reverse genetic and clinical diagnostic applications. Traditional plant breeding involves incorporation of valuable traits from natural variation into agricultural genotypes by hybridization, recombination, and selection. This usually takes many years to achieve. The mutations that drive evolution are rare and random events. Technologies such as gene transformation can be used to improve crop traits by introducing useful foreign DNA. However, transformation often leads to problems such as transgene silencing, species dependence, and a lack of acceptance by consumers. Traditional chemical mutagenesis (forward genetics) was used in plant breeding for many years, based on phenotypic screening. Phenotypic screening for desired traits, however, is not always easy. With the accumulation of large-scale sequence data, emphasis in genomics has shifted from determining gene structure to testing gene function, and this relies on reverse genetic methodology. In contrast to typical reverse genetics techniques such as RNA interference and insertional mutagenesis, TILLING is nontransgenic and generates allelic series of mutations, including knockouts, in the desired gene (Henikoff and Comai, 2003). This technique is applicable to all organisms but is highly suited to plants. The TILLING method is useful for both functional genomics as demonstrated in *Arabidopsis* (McCallum *et al*., 2000b) and crop improvement as demonstrated in wheat (*Triticum aestivum* L.) (Slade *et al*., 2005), maize (*Zea mays* L.) (Till *et al*., 2004); rice (*Oryza sativa* L.) (Wu *et al*., 2005; Till *et al*., 2007); barley (*Hordeum vulgare* L.) (Caldwell *et al*., 2004); sugar beet (*Beta vulgaris* L.) (Hohmann *et al*., 2005); *Lotus japonicus* (Regel) K. Larsen (Perry *et al*., 2003); and soybean (*Glycine max* (L.) Merr.) (Cooper *et al*., 2008).

TILLING produces a large chemically mutagenized population with random mutations across the genome, so that an efficient mutation detection method is essential. SNP discovery methods used in TILLING include full sequencing (McCullum et al., 2000a) denaturing high-pressure liquid chromatography (dHPLC) (Jones, 1999) High Resolution Melting (HRM) point analysis and heteroduplex mismatch cleavage assay using endonuclease CelI followed by sequencing. Among these, the mismatch cleavage assay has high sensitivity in pooled samples, and is therefore high-throughput and low cost.

Materials and Methods

Detection of SNPs in genes of interest, whether induced or endogenous, is a powerful tool to explore gene function and to identify desired mutations for breeding. TILLING has proven to be a valuable methodology for reverse genetics, combining traditional chemical mutagenesis with high-throughput PCR-based mutation detection. The more traditional forward genetics approach relies on the identification of a mutant phenotype followed by the investigation of the causative gene. Mutation breeding has its advantages and limitations. The advantages include creation of new gene alleles that do not exist in germplasm pools and the induction of new gene alleles for a commercial variety so new varieties carrying desired mutation alleles can be directly used as a commercial variety. The limited genetic changes of any single plant of a mutated population and the often recessive nature enable breeders to develop a new variety in a short breeding cycle. The disadvantage of mutation breeding is its limited power in generating the dominant alleles that might be desired. The main points to be considered in the generation of the mutant collection are the choice of the mutagen, the size of the seed population to be mutagenised and subsequently screened and the pooling system to be used to collect the M2 seeds from the M1 plants.

Mutagenesis

In self-pollinating plants, the TILLING method involves chemical mutagenesis (eg., ethylmethane sulfonate (EMS), N-ethyl-N-nitrosourea (ENU), or NaN3), growth of M1 plants and self-fertilization to produce M2 seeds, collection of M2 DNA for screening, and storage of M3 seeds as a bank of mutants and finally crossing of useful mutants for desired traits, which suits a small laboratory and budget. TILLING and Ecotilling are closely related methods useful in rapid detection of small mutations or natural polymorphisms, respectively. These methods rely on the enzymatic cleavage of heteroduplexed DNA with a single strand specific nuclease (i.e., Cel I, Endo I, mungbean nuclease, S1 nuclease, etc.).

The primary requirement for TILLING is a variant population. This can either be created artificially or exploiting the natural diversity. In the first, the artificial creation, make use of the chemical as well as physical muatagenic agents. The most widely used chemical mutagen is EMS. EMS is having advantages over other mutagens ie, it generally produces single nucleotide polymorphisms. The preliminary step for creating a mutant population is identification of the parental line of the crop species and investigates the optimum chemical concentration to be used to induce maximum level of variability. This can be achieved by performing kill curve analysis of the seed population. The period of treatment shall be decided based on the time required to emerge the plumule under the favourable growth conditions. The optimum concentration of mutagen is the concentration at which 50% of seed germination is observed. Above which will be fatal for the seed and may result in very poor germination percentage and field establishment. Typical conditions are 14-18 h in a 30-100 mM (0.3%-1%) EMS solution even though, optimal conditions are dependent on many variables and need to be established empirically for each species. The EMS treated seeds (M1seeds) can be sown directly in the open field or transplant seedlings after germinating in the green house conditions. From the field grown mutants (M1 plants) it could be possible to identify the phenotypic variations, if there is any dominant mutation. The M1 plants were self pollinated to get the homozygosity so that it will be expressed in the next generation (M2 plants). The seeds harvested from the M1 plants (M2 seeds) can be either directly used for DNA extraction or

can be used to raise the M2 population. This population can be used for phenotyping or field evaluation for desired character. A M1 population of 5000 plants will be good for study. To get 5000 plants population should begin with 10,000 M0 seeds for treatment. Induced mutagenesis can be used for creating gene knock-out also.

'TILLING'

The total DNA is extracted following the standard CTAB protocol or Qiagen 96 well DNA extraction kit from 4-5 individual plants of M2 population. A good quality DNA is inevitable for SNP discovery. The next step is the identification of the gene of interest. For the same the available databases can explored to get the sequence. Once the target is fixed, the most apposite region for TILLING could be identified using CODDLE tool (Ref.) depending on the GC content. The optimal length of target sequence that can be TILLED in a single reaction is 1,500 bp. As the preliminary analysis results are available, the primers were designed accordingly to amplify the DNA sequences by PCR. The PCR primers were labeled with fluorescent dyes so that the products can be read in Li-Cor (Ref.). The amplified gene product is cleaved using heteroduplex specific endonuclease enzymes (CEL1, EndoI). Prior to the PCR the DNA samples are pooled eight fold to make the TILLING high through put. More over pooling will enhance the heteroduplex formation. The digested PCR product is purified on sephadex and mixed with formamide dye. Before loading the samples on Li-Cor, were concentrated by vacuum evaporation at 65 ^{0}C in dark. The heat denatured samples were loaded on 6.5% denaturing gel and separated under standard Li-Cor conditions. The resulting images were screened to find out the mutation position and identified mutants were confirmed by sequencing. Further analysis on protein structural variations and functional modifications were predicted using SIFT tool (ref.). The desired mutant can be retrieved from the stored seeds of M2 family and the same can be used as source material in the breeding programme. Using fluorescent labeled primers on a Li-Cor 4300 DNA Analyzer, up to 96 pools, or 768 samples, could be analyzed in a single run, although a subsequent TILLING analysis of each of the samples within a positive pool was necessary to deconvolute the pools and identify the mutated individuals.

A number of bioinformatics tools have been created to aid in the selection of amplicons for TILLING and in the prioritization of the resulting mutations for subsequent analysis. CODDLE (www.proweb.org/coddle/) uses alignments of related sequences and takes into account both the mutagen used and the coding sequence to identify regions of the target gene most likely to generate deleterious mutations; CODDLE sends this information to Primer3 (Rozen and Skaletsky, 2000) to generate suitable primers. GelBuddy (www.proweb.org/gelbuddy/) can be used to automate band calling in the electrophoretic gel images, while PARSESNP (www.proweb.org/parsesnp/) analyses the resulting sequenced mutations and attempts to predict the likely consequence for gene function using a protein homology model.

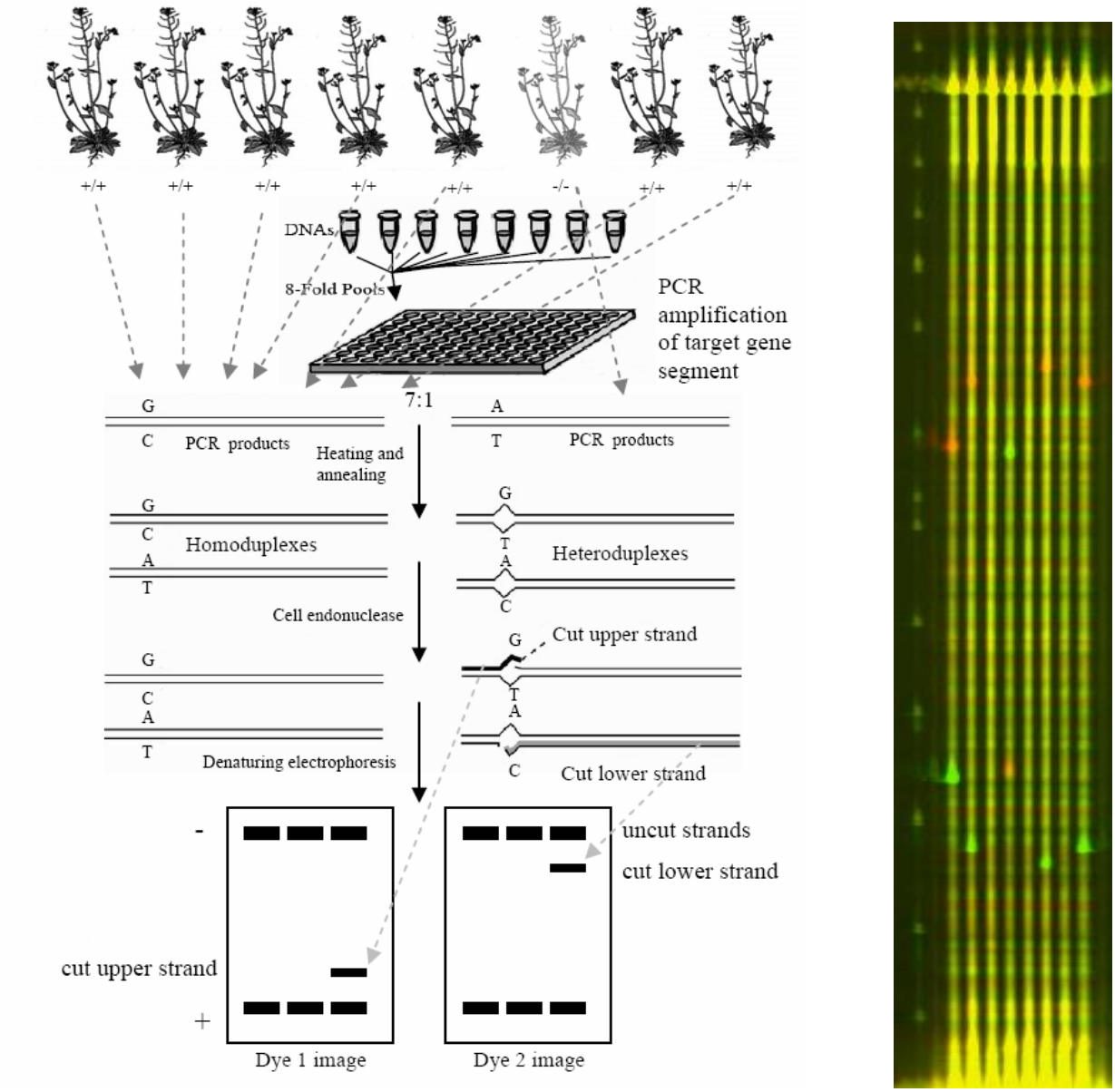

Fig. 1.a. The steps showing the chemical mutagenesis and its discovery using Cel I enzyme.
b. Li-Cor gel photograph showing mutation sites (arrow).

Applications and prospects

EMS creates a larger proportion of non-sense mutations, involving the introduction of novel stop codons, due to the specificity of EMS in creating mainly G–A and C–T transitions, and any individual mutations is therefore more likely to have a phenotypic effect.

Novel DNA sequence information allows the development of additional molecular markers for breeding as well as providing targets for transgenic alteration of gene expression and introduction of new traits. TILLING was developed to take advantage of this new DNA sequence information and to investigate the functions of specific genes. TILLING also shows promise as a

nontransgenic tool to improve domesticated crops by introducing and identifying novel genetic variation in genes that affect key traits. As an alternative to the use of wild varieties, TILLING can be a means to introduce genetic variation in an elite germplasm without the need to acquire variation from exotic cultivars, thus avoiding introduction of agriculturally undesirable traits.

TILLING is accepted and rapidly extended into different crop plants and in animal species. Thus, there now exist TILLING platforms and associated mutagenized populations in *Lotus* (Perry *et al*., 2003), pea (Triques *et al*., 2007), sorghum (Xin *et al*., 2008), soybean (Cooper *et al*., 2008b), oilseeds (Wang *et al*., 2008), bread wheat (Slade *et al*., 2005), barley (Caldwell *et al*., 2004; Talame` *et al*., 2008), rice (Suzuki *et al*., 2008), maize (Till *et al*., 2004), Drosophila (Winkler *et al*., 2005; Cooper *et al*., 2008), and zebrafish (Moens *et al*., 2008).

The TILLING technique was first exploited in the *Arabidopsis* genome project in 2001 (Ref.). In the initial years it self the ATP (*Arabidopsis* TILLING Project) reported over 1000 mutants in more than 100 genes. The comprehensive review is available at www.arabidopsis.org. Perry and colleagues scored around 45600 M2 progenies of 4190 EMS mutagenized M1 plants of *L. japonicus*. They have reported mutants affecting metabolism, morphology and root nodulation (Perry *et al*, 2003). The power of TILLING as a functional genomics tool as well as a crop improvement is reviewed by Slade and Knauf (2005). They have established a TILLING platform to generate large amount of variations in the *waxy* loci of both allohexaploid and allotetraploid wheat in three target genes and they could characterize useful mutants among them. Recently in 2009, a slightly modified TILLING technique for polyploid wheat improvement introduced (Dong *et al*., 2009; Uauy *et al*., 2009). The TILLING can be utilized not only in plant also in animal species also. Kovar *et al*., (2004) demonstrated the use of TILLING/Ecotilling for rapid identification of mutations in mice and TILLING was tested to determine if a known point mutation, *albino (Tyrc)*, a G to C change in the tyrosinase gene, could be detected in genomic DNA. Later in 2006, Gilchrist *et al*., proved successfully the power of the technique in *Caenorhabditis elegans*. They generated an EMS-mutagenised population of approximately 1500 individuals and screened for mutations in 10 genes. A total of 71 mutations were identified by TILLING. Peggy Ozias-Akins and Laura Ramos (2005) explored the potential for inducing mutations in DNA sequence (knockout) specifically to alter the allergen composition and content of peanut seeds. This method of reverse genetics has been widely adopted by the academic community for use in model organisms, including *Drosophila*, zebrafish, and *Arabidopsis* (McCallum *et al*., 2000; Gilchrist *et al*., 2006; Bentley *et al*., 2000; Smits *et al*., 2004; Wienholds et al. 2003; Draper et al. 2004). Improvement of Sugar beet through TILLING is explained under GABI project (Germany). Soybean raffinose synthase genes and microsomal omega-6 fatty acid desaturase genes were screened by Dierking and Bilyeu (2009) for novel alleles in mutagenized soybean populations. They identified new sources of soybean seed meal and oil composition traits through TILLING. The *Brassica* genome project also exploited the possibilities and advantages of TILLING as a functional genomic tool. Stephenson *et al*., (2010) developed the first EMS TILLING resource in the diploid *Brassica* species, *B. rapa*. The mutation density in this population is ~1 per 60 kb, which makes it the most densely mutated diploid organism for which a TILLING population has been published. This resource is publicly available through the RevGenUK reverse genetics platform http://revgenuk.jic.ac.uk. A TILLING

population has been developed for the Danish barley variety Lux (*Hordeum vulgare* L.), by using sodium azide to induce mutations by Lababidi *et al*., (2009). Two of the 13 known dehydrin genes, Dhn12 and Dhn13, respectively, were examined and five independent missense mutations were obtained from a population of 9575 barley mutant plants. Till *et al*., applied the TILLING method to the model crop rice and have identified two different mutagenic treatments that provide a suitably high density of mutations in various target genes (Till et al., 2007). They have established a public TILLING service for maize modeled on the existing Arabidopsis TILLING Project (Till *et al*., 2004). INRA-URGV successfully applied this technique in various crops, such as, pea (Dalmaise *et al*.,-------), allelic variants of melon eiF4E for virus resistance (Nieto *et al*., 2007). Chawade *et al*., (2010) characterized an oat (*Avena sativa*) TILLING population and identified mutations in lignin and β-glucan biosynthesis genes As described above TILLING has been effectively applied in different species as an alternative to transgenics and new species are being added to this array constantly.

The various reports have convincingly shown that TILLING is a technique with considerable potential for crop improvement. It represents an extension of the use of spontaneous and induced mutants in plant breeding and allows the direct identification of beneficial nucleotide and amino acid changes in genes with known functions and their use as the genetic markers for selection. The range of alleles that can be developed via TILLING in a short time is unparalleled and unlikely to be found elsewhere in the pool of germplasm accessible to plant breeders (including landraces and undomesticated relatives). Because the TILLING population is a permanent resource, the results of basic scientific research can be efficiently translated into crop improvement as new information about the functions of potential gene targets becomes available.

Mutagenized populations can be created at relatively low cost, although diploid species that are intolerant of high mutation frequencies require much larger populations for full coverage. Conversely, highly-mutagenized lines of polyploidy species may require significant backcrossing to remove extraneous mutations before they can be assessed for phenotypes or used in plant breeding. Importantly, the use of such novel alleles in crops will not be impeded by the tough regulatory regimes that cover GM crops; this alone should assure the rapid deployment of this technology in plant breeding. For agronomic end use of a variety acquired through TILLING, a number of backcrosses (four or more) may also be required to purge potentially undesirable background mutations. When backcrossing, the removal of background mutations can be accelerated through marker-assisted selection using the SNP itself as a molecular marker.

References:

1. Bentley A, MacLennan B, Calvo J, Dearolf C: Targeted recovery of mutations in Drosophila**.** *Genetics* 2000, 156(3):1169-1173.

2. Caldwell, D.G., N. McCallum, P. Shaw, G.J. Muehlbauer, D.F. Marshall, and R. Waugh. 2004. A structured mutant population for forward and reverse genetics in Barley (*Hordeum vulgare* L.). Plant J. 40:143–150.

3. Chawade *et al*., 2010

4. Cooper, J.L., B.J. Till, R.G. Laport, M.C. Darlow, J.M. Kleff ner, A. Jamai, T. El-Mellouki, S. Liu, R. Ritchie, N. Nielsen, K.D. Bilyeu, K. Meksem, L. Comai, and S. Henikoff . 2008. TILLING to detect induced mutations in soybean. BMC Plant Biol. 8:9.

5. Dalmais M, Schmidt J, Le Signor C, Moussy F, Burstin J, Savois V, Aubert G, Brunaud V, de Oliveira Y, Guichard C, Thompson R, Bendahmane A: UTILLdb, a *Pisum sativum* in silico forward and reverse genetics tool. *Genome Biology* 2008, 9:R43.

6. Dierking and Bilyeu 2009

7. Dong C, Dalton-Morgan J, Vincent K, Sharp P: A Modified TILLING Method for Wheat Breeding. *Plant Gen* 2009, 2:39-47.

8. Draper et al. 2004.

9. Gilchrist EJ, O'Neil NJ, Rose AM, Zetka MC, Haughn GW: TILLING is an effective reverse genetics technique for Caenorhabditis elegans. BMC Genomics 2006, 7:262.

10. Henikoff, S., and L. Comai. 2003. Single-nucleotide mutations for plant functional genomics. Annu. Rev. Plant Biol. 54:375–401.

11. Hohmann, U., G. Jacobs, and C. Jung. 2005. An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. Plant Breed. 124:317–321.

12. Jones, A.C. et al. Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. *Clin. Chem.* 45, 1133–1140 (1999).

13. Khush, 2001

14. Kovar *et al*., 2004

15. McCallum, C.M., L. Comai, E.A. Greene, and S. Henikoff . 2000a. Targeted screening for induced mutations. Nat. Biotechnol. 18:455–457.

16. McCallum, C.M., L. Comai, E.A. Greene, and S. Henikoff . 2000b. Targeting Induced Local Lesions IN Genomes (TILLING) for plant functional genomics. Plant Physiol. 123:439–442.

17. Moens *et al*., 2008

18. Nieto *et al*., 2007

19. Peggy Ozias-Akins and Laura Ramos 2005

20. Perry, J.A., T.L. Wang, T.J. Welham, S. Gardner, J.M. Pike, S. Yoshida, and M. Parniske. 2003. A TILLING reverse genetics tool and a web accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiol. 131:866–871.

21. Rozen S, Skaletsky H: Primer 3 on the WWW for general users and for biologist programmers. Bioinformatics Methods and Protocols: Methods in Molecular Biology Cambridge: Humana PressKrawetz S, Misener S 2000, 365-386.

22. Slade and Knauf 2005

23. Slade, A.J., S.I. Fuerstenberg, D. Loeffl er, M.N. Steine, and D. Facciotti. 2005. A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. Nat. Biotechnol. 23:75–81.

24. Smits BMG, Mudde J, Plasterk RHA, Cuppen E: Target-selected mutagenesis of the rat. *Genomics* 2004, 83:332-334.

25. Stephenson *et al.*, 2010

26. Suzuki T, Eiguchi M, Kumamaru T, Satoh H, Matsusaka H, Moriguchi K, Nagato Y, Kurata N: MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice**.** *Mol Genet Genomics* 2008, 279(3):213-223.

27. Talamè V, Bovina R, Sanguineti MC, Tuberosa R, Lundqvist U, Salvi S: TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotechnology J* 2008, 6:477-485.

28. Till, B.J., J. Cooper, T.H. Tai, P. Colowit, E.A. Greene, S. Henikoff , and L. Comai. 2007. Discovery of chemically induced mutations in rice by TILLING. BMC Plant Biol. 7:19.

29. Till, B.J., S.H. Reynolds, C. Weil, N. Springer, C. Burtner, K. Young, E. Bowers, C.A. Codomo, L.C. Enns, A.R. Odden, E.A. Greene, L. Comai, and S. Henikoff . 2004. Discovery of induced point mutations in maize genes by TILLING. BMC Plant Biol. 4:12.

30. Triques *et al*., 2007

31. Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J: A NAC Gene Regulating Senescence Improves Grain Protein, Zinc, and Iron Content in Wheat. *Science* 2006, 314:1298-1301.

32. Wang N, Wang Y, Tian F, King GJ, Zhang C, Long Y, Shi L, Meng J: A functional genomics resource for Brassica napus: development of an EMS mutagenized population and discovery of FAE1 point mutations by TILLING. New Phytol 2008, 180(4):751-765.

33. Wienholds et al. 2003

34. Winkler *et al*., 2005

35. Wu, J.L., C. Wu, C. Lei, M. Baraoidan, A. Bordeos, M.R.S. Madamba, M. Ramos-Pamplona, R. Mauleon, A. Portugal, V.J. Ulat, R. Bruskiewich, G. Wang, J. Leach, G. Khush, and H. Leung. 2005. Chemicaland irradiation-induced mutants of indica rice IR64 for forward and reverse genetics. Plant Mol. Biol. 59:85–97.

36. FAO: Food and Agriculture Organization**.** *ProdSTAT; FAOSTAT* 2008.