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Molecular TILLING and EcoTILLING: Effective Tools for Mutant Gene Detection in Plants

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ABSTRACT

Forward genetics has been responsible for our understanding of many biological processes and is an excellent method for identifying genes that function in a particular process. In reverse genetics, the functional study of a gene starts with the gene sequence rather than a mutant phenotype. Using various techniques, a gene's function is altered and the effect on the development and phenotypic traits of the plant is analysed. Reverse genetics is an important complement to forward genetics. For example, using reverse genetics, one can investigate the function of all genes in a gene family, something not easily done with forward genetics. Further, one can study the function of a gene found to be involved in a process of interest in another species, but for which no forward genetic mutants have yet been identified. Finally, the vast majority of genes have not yet been mutated in most plants and reverse genetics allows their study. The availability of complete genome sequences combined with reverse genetics can allow every gene to be studied. In this chapter we review the recent progress that has been made towards identifying induced point mutations and natural variation in different plants using the high throughput reverse genetics technologies, TILLING and EcoTILLING, and discuss the prospects of using these techniques in ornamentals.

Keywords: TILLING, EcoTILLING, reverse genetics, single nucleotide polymorphisms, gene mutation, plants
Abbreviations: EMS, Ethyl methyl sulfonate; **IRD**, Infrared dye; **kb**, kilobase pair; **Mb**, Megabase pair; **mM**, millimolar; **SNP**, single nucleotide polymorphism; **TILLING**, **T**argeting **I**nduced **L**ocal **L**esions **IN** **G**enomes

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INTRODUCTION

Genetic variation exists naturally or may be generated by mutagenesis techniques. The resulting variation, whether expressed in the phenotype or not, exists in DNA sequences. Identifying variation in genes is a means of adding value to genetic resources which can be associated with phenotypic change. Improvement of any plant depends on the understanding of the plant biology, the interactions between genes and environments, the genetic basis of allelic variation, and the resultant plant phenotype. The availability of diverse genetic variation helps breeders to make decisions on proper use of valuable genetic resources to meet current and future challenges, which include those posed by biotic and abiotic stresses, the need for improved nutritional and functional properties, the requirement for wider adaptability, and the increased demand for sustainable food production.

The most common form of genetic variation in natural

Received: 10 August, 2007. Accepted: 4 October, 2007.

Invited Review

or induced populations is due to single nucleotide change, insertion and deletion, which generates single nucleotide polymorphisms (SNPs). For decades, breeders and geneticists have studied the genetics behind phenotypic variation by identifying genes and their functional products. This is known as the forward genetics approach. Determination of functional product is limited by the need to isolate and study functionally defective mutants of the specific process of interest (Gilchrist and Haughn 2005). Reverse genetics approaches, however, allow investigating the function of known and unknown genes by disruption or alteration of a particular gene.

With the availability of whole genome sequences of different plant species, researchers have a set of tools to potentially understand the functions of thousands of newly identified genes in the perspective of the whole organism. This may facilitate the identification of novel genetic variations in any gene affecting the phenotype of an important trait, the development of additional new markers, the understanding of non-transgenic gene expression, and the introduction of novel traits into specific plants (Slade and Knauf 2005; Synäven 2005). Because the identification of natural or induced variation in a target gene is crucial for plant improvement, an efficient cost-effective SNP genotyping method is required for routine application.

In plants, several reverse genetic strategies have been used to provide targeted inactivation of gene function. These approaches included transposon mutagenesis (Tissier *et al*. 1999), RNA-mediated gene silencing or RNA interference (Voinnet 2002), post transcriptional gene silencing (Chuang and Meyerowitz 2000), and insertional mutagenesis (Alonso *et al*. 2003). All of these strategies are efficient, however, they have disadvantages which limited their use in functional genomics and they are not equally applicable to all organisms (Hanikoff and Comai 2003). The development of novel technologies for an effective general reverse genetic strategy is essential for the functional characterization of a large number of genes.

Targeting **I**nduced **L**ocal **L**esions **IN** **G**enomes (TILLING) is a powerful high throughput technique which identifies single base changes in a specific gene in a mutagenized population (McCallum *et al*. 2000a, 2000b; Till *et al*. 2003a, 2003b; Gilchchrist and Haugnh 2005; Comai and Henikoff 2006; Haughn and Gilchchrist 2006; Till *et al*. 2006). An expansion of the TILLING technique is Eco TILLING, which can be used to discover point mutations or polymorphisms in natural populations (Comai *et al*. 2004). These techniques allow identifying diverse versions of genes in a gene pool or germplasm and acquiring information about their gene function. This information can provide guidelines to develop new strategies for genetic improvement of plants. Identifying novel mutations can provide new opportunities for genetically manipulating and enhancing the performance of plants and increasing the discovery of markers linked to specific traits or genes of interest.

TILLING and EcoTILLING also can be used to identify unknown and known point mutations from a set of candidate genes. Both methodologies provide proof of function for both natural and induced variations. Reverse genetics techniques allow recognition of possible loss of function for a particular gene during early stages of development (Stemple 2004).

TILLING AND ECOTILLING PROCEDURES

Identifying rare mutations or polymorphisms is a laborious, time consuming and expensive undertaking, as it requires the screening of large populations with thousands of individual plants. Consequently a reliable, less expensive and high throughput screening technology is essential for practical use. Several gel based assays are available such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), constant denaturant gel electrophoresis (CDGE) and single strand conformational polymorphism (SSCP) (Gross *et al*. 1999; de Francesco and Perkel 2001; Hestekin *et al*. 2006). The disadvantages of these methods are that they can neither determine the type of polymorphism in the DNA fragment nor can they pinpoint the location of the mutation (Gilchrist *et al*. 2006), and all of them only work efficiently with small DNA fragments of 100-300 base pairs (de Francesco and Perkel 2001).

With TILLING, DNA samples from thousands of mutated individuals can be screened in a high throughput fashion to identify single nucleotide polymorphisms. McCallum *et al*. (2000a) first developed the protocol to screen EMS-induced point mutations in plants. Following the discovery of an effective single strand mismatch specific cleavage enzyme, celery nuclease Cel I (Oleykowski *et al*. 1998), Colbert *et al.* (2001) successfully adapted the TILLING technique for high throughput mutation discovery using a mutagenized Arabidopsis population with a LI-COR gel analyzer system (LI-COR Biosciences, Lincoln, NE, USA). CODDLE (Choosing Codons to Optimize Discovery of Deleterious LEsions) (http://www.proweb.org/input/) is a web based program used to identify the regions which would have the highest probability of generating deleterious mutations by EMS mutagenesis, to till within a target gene. They designed several sets of forward and reverse primers for PCR using the publicly available program, Primer3 (Rozen and Skaletsky 2000). After mutations have been identified and sequenced, the program Project Aligned Related Sequences and Evaluate SNPs (PARSESNP) can be used to analyze the polymorphisms in genes and predict the severity of missense changes (Taylor and Greene 2003).

The basic TILLING and EcoTILLING techniques involve the following sequential steps: DNA extraction and quantification; DNA normalization and pooling; PCR amplification of the pooled DNA for target region with gene specific primers; heteroduplex formation by denaturation and renaturation; cleavage of the PCR products by mismatch-specific celery nuclease Cel I; and identification of the cleaved products on denaturing polyacrylamide gel (Till *et al*. 2003a). An overview of the TILLING and EcoTILLING processes is diagramed in **Fig. 1**. Genomic DNA isolated from mutagenized samples can be pooled with up to eight individuals and arrayed in micro titer plates. Equal amounts of DNA from each individual sample should be used to comprise the pool. Although the pooling of DNA samples would increase the efficiency of screening by reducing time, cost and labor, however, if the concentration of any DNA sample is lower than others, mutations within the pool might not be detected. The pooled DNA samples are amplified by PCR using gene specific primers which are designed to amplify 1-1.5 kilobase (kb) fragments and are end labeled with two different fluorescent dyes. Using differently labeled forward and reverse primers allows loading of the cleaved products in the same gel and tracking of the electrophoresis in two channels (Colbert *et al*. 2001; Till *et al*. 2003a, 2003b). This makes comparison straight forward with simultaneous detection of a mutation on the complementary strand, and pinpointing of the precise mismatch position. It also corrects for the appearance of artificial primer-dimer bands which appear in both channels. The amplified PCR products are denatured and re-natured by heating and cooling to form a heteroduplex (Till *et al*. 2003a, 2003b; Comai *et al*. 2004; Gilchrist and Haughn 2005). If the pooled DNA contains a single nucleotide mutation in the target sequence, the heteroduplex between wild and mutant alleles is cleaved precisely at the 3′ end of the mismatch by the S1 family single strand specific endonuclease Cel I (Oleykowski *et al*. 1998), leaving the wild type duplex intact. The cleaved products can be separated easily by either 10% polyacrylamide gel followed by staining with SYBR Green (Yang *et al*. 2004) or an ABI377 sequencer (Perry *et al*. 2003) or Transgenomic WAVE High Sensitivity denaturing High Performance Liquid Chromatography (WAVE-HS dHPLC) (Caldwell *et al*. 2004) or capillary DNA analyzer (Suzuki *et al*. 2005) or the most commonly used apparatus LI-COR IR^2 gel analyzer (Colbert *et al*.

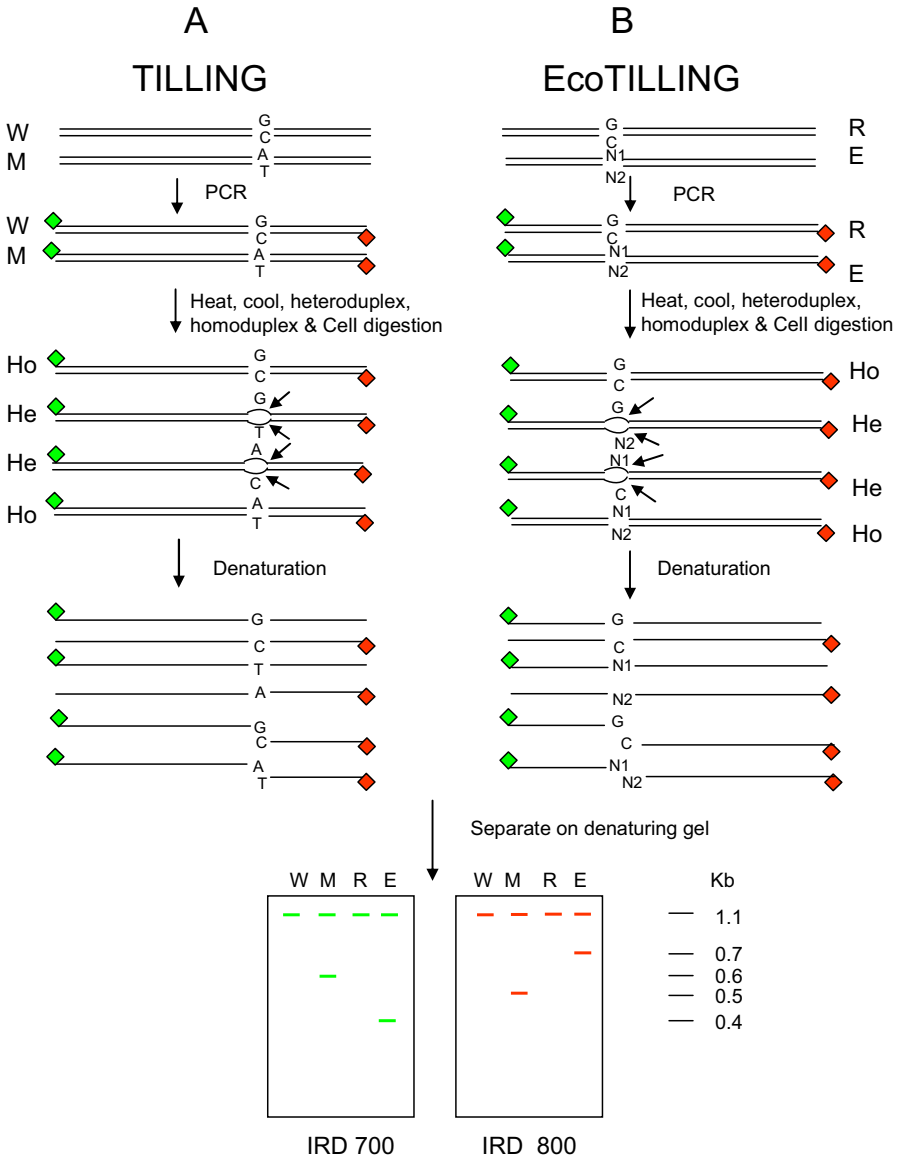

Fig. 1 The TILLING and EcoTILLING procedures. (**A**) TILLING: PCR amplification of wild type (W) and Mutant (M) DNA sample. (**B**) EcoTILLING: PCR amplification of Reference (R) and Ecotype (E) DNA sample. The gene-specific forward and reverse primers 5′-end labelled with IRD700 and IRD800 are identified with green and red rectangles, respectively. After PCR, the PCR products are heated and cooled to form homoduplex (Ho) and heteroduplex (He), followed by CEL I digestion. Heteroduplex DNA will be cleaved at the 3′ end of the mismatch (indicated by arrow). The homoduplex DNA will be intact. The cleaved products are detected by polyacrylamide denaturing LI-COR gels in both IRD 700 and 800 channels. The size of the cleaved fragments in both channels adds up to the size of the PCR fragment (homoduplex fragment) and also specifies the location of the mutation within the targeted gene.

2001; Till *et al*. 2003a, 2003b; Comai *et al*. 2004; Henikoff *et al*. 2004; Till *et al*. 2004a, 2004b; Gilchrist *et al*. 2006; Till *et al*. 2006, 2007; Weil and Monde 2007).

MUTANT GENERATION FOR TILLING

Successful TILLING of any species relies on the frequency of the induced mutations in the population to be screened. This entirely depends on the exchange between the effect of the mutagen on the germinal tissue and the side effects (toxicity and/or sterility) of the mutagen (Till *et al*. 2003b). A wide variety of physical, chemical and biological mutagenic methods can be used to generate genetic alterations. The most commonly used mutagenic agents are chemical classes such as alkylating agents, including Ethyl methyl sulfonate (EMS) and N-ethyl-N-nitrosourea (ENU); deaminating agents, including Nitrous and Nitroso guanidine; hydroxylating agents, including hydroxylamine; and other chemicals with unkown mechanism of action such as Diepoxybutane (DEB) and sodium azide (Waugh *et al*. 2006).

EMS is an extremely efficient mutagen that is commonly used in plants to create mutant populations. EMS produces random mutations in genetic material by nucleotide substitution; specifically by guanine alkylation. This typically produces only point mutations in the DNA sequence or genome. This mutagen induces a large number of recessive mutations in plants (Gilchrist and Haughan 2005). McCallum *et al*. (2000a) first developed TILLING to screen EMS-induced point mutations in *Arabidopsis thaliana*. They soaked seeds in 20-40 millimolar (mM) EMS for 10-20 hours (McCallum 2000a; Greene *et al*. 2003), and grew them to obtain M1 plants. Since M1 plants are chimeric for mutations, they self fertilized M1s to produce M2 lines for DNA isolation. Extracted DNA samples were arrayed in 96-well plates and pooled up to eightfold for TILLING (Colbert *et al*. 2001). EMS alkylates guanine bases and the alkylated guanine can be mis-matched with T instead of C to result in a G/C to A/T transition (Ashburner 1989; Henikoff and Comai 2003; Greene *et al*. 2003; Stemple 2004). This type of mutation is difficult to identify by forward genetic approach (Greene *et al*. 2003) or by any other reverse genetic approach (McCallum *et al*. 2000a, 2000b).

The use of chemical mutagenesis was restricted to forward genetics due to unavailability of a high throughput technique for identifying point mutations (Gilchrist and Haughn 2005), but it is essential in reverse genetics where identification of a change in sequence is the way to identify a mutant (Alonso and Ecker 2006). The EMS induced point mutation can be identified by the TILLING technique. This is a high throughput approach which can now be used to detect single base substitution in a target gene or to genotype single nucleotide polymorphisms (SNPs).

One of the challenges for chemical mutagenesis is to determine an ideal dose of mutagen, which requires a considerable amount of trial and error (Henikoff and Comai 2003). The main drawback of chemical mutagenesis is the difficulty in identifying the mutant phenotype caused by a single base change in a large genome (Alonso and Ecker

2006). In addition, the resulting damage caused by the mutagen can be severe. If a mutation causes deletion, insertion and rearrangement, the mutated allele can lose its function. If the mutation is a single base change, it can lead to a broader range of effects such as loss or gain of function, or even novel gene function (Alonso and Ecker 2006).

The effect of mutagenesis can be homozygous or heterozygous. Homozygous mutation of a gene can lead to lethality or sterility. The most desirable mutation is heterozygous. The chemical mutagen EMS can induce single base changes in coding regions resulting in silent, nonsense and/or missense mutations. The silent mutation does not alter any encoded amino acid so the effect will be silent, whereas missense and nonsense mutations are deleterious. While a missense mutation will change the amino acid in the protein by a particular codon change, a nonsense mutation may result in a premature stop codon, or a nonsense codon in the transcribed mRNA, and possibly a truncated, and often nonfunctional protein product (McCallum *et al*. 2000a, 2000b). Since creating mutant populations is particularly difficult for vegetatively propagated crops and plants requiring long time for reproduction, EcoTILLING is the method of choice to identify natural variation in these plants (Slade and Knauf 2005).

TILLING AND ECOTILLING IN DIFFERENT CROPS

TILLING and EcoTILLING are non-transgenic techniques for identifying induced and natural genetic variations, respectively. They are sensitive, rapid and efficient methods for point mutation detection. The TILLING method was originally developed for use in the fully sequenced model plant, *Arabidopsis thaliana*, for functional genomics and identifying mutations. Successful applications of the TILLING technique for functional genomics have been established in Arabidopsis, *Drosophila* and zebrafish (Henikoff and Comai 2003; Henikoff *et al*. 2004; Stemple 2004). Recently, application of this method has extended to other non-model plants and crops. We review here the application and current status of TILLING and EcoTILLING technologies in various plants and crops including Arabidopsis, *Lotus*, wheat, maize, rice, barley, soybean, tomato, and sugarcane.

Current status of TILLING

Arabidopsis

The TILLING technique was first used in *A. thaliana* to detect previously known and unknown mutations. Till and his colleagues (2001) established TILLING as a service called the Seattle TILLING project (STP) (formerly the Arabidopsis TILLING project (ATP)) to rapidly deliver an allelic series of EMS induced mutations in target 1 kb loci (Till *et al*. 2003b). The ATP was a joint effort between Comai's laboratory (University of Washington) and Henikoff's laboratory (Fred Hutchinson Cancer Research Centre, Seattle, WA). The Arabidopsis TILLING Project found on average 10 mutations per kb in about 3000 EMS mutagenized plants. They identified 1890 mutations in the 192 genes screened. The EMS mutagen generates over 99% G/C to A/T transition mutations (Greene *et al*. 2003). Gilchrist and Haughn (2005) suggested that the screening population could be expanded to up to 6912 individuals if no deleterious mutation is detected in the initial screening of 3000 plants. A total of 7451 mutations have been reported from the Arabidiopsis TILLING Project (ATP highlights, http://TILLING fhcrc. org:9366/arab/status.html, as of September 31 2007). The developed TILLING technique is highly sensitive and powerful and can be used efficiently for small or large scale or even in genome wide discovery of point mutations (Mc Callum *et al*. 2000b; Alonso and Ecker 2006).

McCallum *et al*. (2000a) used the TILLING method to screen independent mutations in Chromomythylase 2 (*CMT2*) and Chromomythylase 3 (*CMT3*) genes and identified four mutated individuals from 835 M2 lines. Among them one mutant line changed a CAG Glu codon to a TAG stop codon which truncated the *CMT3*, which thus lacked the four conserved blocks which are essential for chromomythylase enzyme function. The other three individual mutants are homozygous for the *CMT3* knockout in the *CMT1* null ecotypes, demonstrating that loss of function for at least two chromomythaylase is compatible with viability. Recently, this technique has been used successfully to discover point mutations in several other plants including *Lotus*, maize, wheat, rice, soybean, canola and poplar.

Lotus japonicus

The gene functions for root symbiosis with rhizobia and arbuscular mycorrhiza fungi, compound leaf development, flower development and pereniality cannot be studied with the model organism, Arabidopsis (Perry *et al*. 2003). Perry and colleagues established a Lotus TILLING facility at the John Innes Centre, UK for investigating the legume-rhizobium symbiosis for the model legume *Lotus japonicus* to detect point mutations in any gene of interest within a large EMS mutagenized population. They developed and scored 45,600 M2 progenies of 4190 EMS mutagenized plants and identified a number of different individual plants carrying functionally defective mutant allelic genes which affect metabolism, morphology and root nodule symbiosis. Symbiosis Receptor-like Kinase (*SYMRK*) gene was fond to be essential for both mycorrhiza and rhizobial symbiosis in *Lotus japonicus*. Mutations in lotus *SYMRK* genes were previously identified and individuals carrying mutations of the gene were incapable of forming root nodules and arbuscular mycorrhiza (Stracke *et al*. 2002). Perry and colleagues (2003) used 5472 kb *SYMRK* gene genomic sequences for TILLING. They designed 3 primer combinations to cover the protein kinase region. Several mutants in the protein kinase domain of *SMYRK* were identified. Several non nodulating plants with mutations in alleles such as *SL605*, *SL391*, *SL1951*, *SL160*, *SL140* and *SL3472-2* were found as well. Except *SL140* and *SL3472*, the entire non-nodulating mutant population set seed (Perry *et al*. 2003). All of the mutants replaced G with A which is consistent with predominant G/C to A/T transition induced by EMS (Anderson 1995).

Sucrose synthase (*SUS*) is an essential plant metabolic enzyme since it cleaves sucrose to fructose and UDP-glucose to facilitate sucrose utilization. There are three isoforms of *SUS* in Lotus, *SUS1*, *SUS2*, and *SUS3*. Welham *et al*. (2005) used TILLING to identify individuals possessing point mutations in *SUS* gene isoforms. They found a number of mutations in each isoform. The mutation rate was 3 times higher in *SUS1* than *SUS2* per kb genomic region. Among all identified mutants, 10 alleles for *SUS1* were predicted to possess deleterious mutations. The TILLING population had an average of 1302 mutations per genome. Among them 64% of the mutations resulted in a missense change and 35% caused silent changes (http://www.lotusjaponicus.org/TILLINGpages/Developments.htm).

Maize

A maize TILLING facility has been established in Purdue University with the help of the Seattle TILLING project (http://genome.perdue.edu/maizeTILLING). The maize genomic sequence has not been completed yet. The procedure for maize TILLING is identical to Arabidopsis TILLING. The only difference is that the maize TILLING population was created by treating pollen instead of seed with EMS. Although maize has a genome that is about 20 times larger than that of Arabidopsis, the TILLING result is similar to Arabidopsis. The mutation rate in maize is lower than that detected in Arabidopsis (Till *et al*. 2004b). Because of the lower rate of mutation in maize it requires the screening of about 4000 mutagenized lines to obtain a comparable mutation series as in about 2300 lines in Arabidopsis. Till *et al*.

(2004b) screened 700 pollen mutagenized plants using 11 different genes and obtained 10 missense and seven silent mutations but no truncation mutant. Although the mutagenized population was small and the rate of mutation was low, some useful mutations were identified. Among 11 genes screened they discovered an excellent promising allelic series for one of the chromomythylase genes, *DMT102*, with 3 missense mutations. These mutations can cause significant damage to the protein. The allelic series of *DMT102* can now be used for understanding the connection between DNA methylation, chromatin structure, SiRNAs and transposon biology in maize (Till *et al*. 2004b). The maize TILLING facility has already discovered 319 mutations in 62 genes which cover about 76kb of genomic sequence and 47 kb of exons. Heterozygous mutations were identified as well (Weil and Monde 2007). Two mutant maize populations were developed for TILLING from B73 and W22 inbred lines. The B73 and W22 populations have 2370 and 1276 unique mutant lines, respectively. The mutation rate in the B73 was 0.93 and in the W22 was 2.1 per kb in 1000 families (Weil and Monde 2007). The maize genome was estimated to contain 35,000 to 41,000 genes and on average each maize gene contains 945 bp of protein coding exon with 0.71 to 0.83 damaging alleles per gene (Weil and Monde 2007). They developed two additional populations from W22 and B73 and preliminary TILLING tests indicated that the mutation rate of each population is roughly 2-4 times higher than the population reported earlier (Weil and Monde 2007).

Wheat

TILLING technology has been applied successfully to wheat, which has an allohexaploid genome that is 140 times larger than the Arabidopsis genome (Slade *et al*. 2005). The wheat genome is derived from three diploid genomes of closely related ancestors. Consequently any desirable genetic changes are difficult to detect because most of the wheat genes have redundant copies in the hexaploid wheat genome (Slade *et al*. 2005). Slade and colleagues (2005) studied the granule bound starch synthase 1 (*GBSSI*) or waxy starch gene. With only one or two functional *GBSSI* genes wheat produces starch with intermediate levels of amylopectin. Amylopectin has economically valuable functional qualities and unique physiochemical properties. They identified a total of 246 novel alleles of waxy genes out of 1920 EMS mutagenized lines from hexa- and tetraploid wheat. The frequency of mutation determined for about 800 M2 plants from several regions of the genome was about 1 mutation per 24 Kb and 40kb in hexaploid and tetraploid wheat, respectively. The identified allelic series were due to multiple truncations, splice junctions and missense mutations. Eighty four missense mutations were found among 246 new alleles and 54 of the 84 mutations were predicted to severely affect protein function. Favourable allelic variants for a limited number of loci obtained within the same background can be combined by marker-assisted backcrossing within few generations (Bagge *et al.* 2007). The identifIcation of this allelic series and the generation of many useful novel mutations in the waxy loci of wheat demonstrated the potential of using TILLING for polyploid crop improvement.

Rice

Rice is the staple food for more than half of the world's population. Rice has been used as a model crop for cereals as it has a relatively small genome of about 430Mb (Mega bases). There are two major subspecies of cultivated rice, *Oryza sativa* ssp. *japonica* and ssp. *indica*. The rice genome has been sequenced and was estimated to contain 50, 000 genes (Yu *et al*. 2002; Goff *et al*. 2002). The TILLING technique has been applied to rice for developing a high throughput system to identify mutations. However, developing a well mutagenized population of rice is challenging due to the nature of this species. The response to mutagenic treatment is varied even within the same species (Wu *et al*. 2005). Efficient TILLING requires a population with ≥ 1 mutation/500kb. IR4 is the most widely grown indica rice cultivar in Asia and was used to develop a mutation population because of its wide adaptability and valuable agronomic characteristics (Wu *et al*. 2005). Wu *et al*. (2005) screened 2000 individuals from a population mutated with 0.8% and 1.0% EMS. From 10 genes they identified with TILLING, they detected two independent mutations in two genes, *pp2A4* encoding a serine/threonine protein phosphatase catalytic subunit, and *C7* encoding a callose synthase, with a mutation rate of 0.5 mutations per Mb. Different concentrations of EMS mutagen were used to increase the mutation rate. A 1.6% EMS concentration generated the highest frequency of mutations, 1 mutation per Mb. However, albino (8.7%) was the most frequently observed phenotype (Wu *et al*. 2005). Till *et al*. (2007) reported the generation of two mutant libraries from the sub-species japonica cultivar, Nipponbare, using two different mutagens, 1.5% EMS and a combination of 1mM sodium azide plus 15mM methyl nitrosourea (AZ-MNU). A total of 57 mutations were identified in 10 targeted genes, of which 27 mutations were identified from EMS and 30 from the AZ-MNU treated populations. The estimated mutation rate was 1 mutation/294 kb for 1.5% EMS and 1 mutation/265 kb for the AZ-MNU treated population. The mutation rate was much higher than previously reported (Wu *et al*. 2005). Among 10 target genes the mutation in the *OsDREB* allelic series predicted severe loss of function due to truncation mutation. The allelic series also had six missense changes which were predicted to affect protein function (Till *et al*. 2007). The EMS mutagenized rice populations have 70% GC>TA, 11% AT>GC, 4%GC>TA and 15% AT>TA exchanges which is consistent with the mutational spectrum reported in barley (Caldwell *et al*. 2004) but differs from Arabidopsis, maize and wheat (Till *et al*. 2007). The created mutation population is suitable for high throughput TILLING as the mutation density is comparable to other plants used for public TILLING (Till *et al*. 2007).

Barley

Barley is a diploid monocotyledon with a large genome size of about 5300 Mb. With the availability of genomics based resources a number of candidate genes for various traits have been identified. Due to the large genome size and lack of simple and high throughput transformation systems for Triticeae, an efficient simple high throughput system is required for confirmation and validation of gene function (Caldwell *et al*. 2004). Two chemically mutagenized populations were developed in barley using 20-30 mM doses of EMS and comprised of 9216 plants (Barley TILLING, Dundee, Scotland, http://germinate.scri.sari.ac.uk/barley/mutants/). These were used to study gene function by high throughput TILLING using Hordoinline-a (*Hin-a*) and the *Hordeum vulgare* Floral Organ regulator-1 (*HvFor1*) genes (Caldwell *et al*. 2004). Following PCR and heteroduplex cleavage with Cel I they used a post-digestion intercalating dye labelling system to prevent exonucleolytic activity of Cel I enzyme (Caldwell *et al*. 2004). The enzyme may cleave the fluorophores from the fluorescently labelled amplified product which limits the detection of unknown signal of nucleotide polymorphism (Henikoff and Comai 2003). Caldwell *et al* (2004) screened the mutant populations and identified six induced transition alleles of *HvFor1* and *4* induced alleles (two transition and two transversion) of the *Hin-a* genes. The two transversion mutant alleles of *Hin-a* genes were new and did not match with other previously identified SNPs within the *Hin-a* gene. The induced mutation frequency was about one per million bp or 5000 per genome based on screening data of *Hin-a* and *HvFor1* candidate genes. The observed frequency is notably lower than the observed frequency in Arabidopsis. However, the results demonstrated the utility of reverse genetic approach for identi-

fying mutations. The derived EMS mutant population may be useful for functional genomic research (Caldwell *et al*. 2004).

TILLING techniques also have been developed in different laboratories in diverse crops. The Canadian TILLING Initiative (Can-Till) was started by George Haughn and Erin Gilchrist, University of British Columbia, Vancouver, BC, Canada.

The Can-Till project (http://www.botany.ubc.ca/can-till) has established TILLING in *A. thaliana*, *Caenorhabditis elegans*, and *Brassica oleracea*. In *B. oleracea* mutations were identified and predicted to play a role in abiotic stresses such as temperature, drought and nutrient imbalance. Currently Can-Till is focused on establishing the TILLING technique for the oilseed crop *B. napus* to develop canola with optimal seed coat characteristic and reduced anti-nutritional factors.

Arcadia Biosciences (Davis, CA, USA) developed TILLING technology for *Triticum aestivum* and *T. durum* (Slade *et al*. 2005) and the company is using TILLING technology to develop a processing tomato with higher lycopene and other antioxidants and higher solids content than conventional varieties. In addition, this company also is applying TILLING to increase shelf life in lettuce, strawberry, melon and tomato (http://www.arcadiabio.com/). In 100 EMS mutagenized tomato plants Carriero *et al*. (2004) identified 6 mutations in the *rab11a* gene, which is thought to be involved in the control of protein trafficking in the cell. Mc Callum *et al*. (2003) screened 1300 M2 individuals and identified two missense alleles in tomato that are predicted to affect the loss of fruit ripening enzyme polygalacturonase function.

TILLING has been applied to sugar beet, leading to the identification of five deficient bolting gene mutants from EMS mutagenized populations (Hohmann *et al*. 2005).

Mitchum and Meksem (2005) applied TILLING in two candidate SCN resistance genes, *Rhg1* and *Rhg4*, in soybean. They have developed populations from the soybean cyst nematode resistant cultivar, Forest, for mutation analysis and identified up to three mutations per 1.5 kb targeted sequence from 768 M2 plants. The alteration of SCN resistance was confirmed by phenotypic and genotypic analysis. Several mutants were identified by TILLING within the LRR-RLK (Leucine Rich Repeat Receptor Like Kinase) candidate *Rhg4* gene. However, mutant Q263 did not show any loss of function.

The Legume Genetics and Ecophysiology Research unit (UR-LEG, INRA-URGV, Evry, INRA Montpellier, and JIC Norwich) generated publicly available TILLING and Eco TILLING resources for *Medicago tranculata* which contains EMS mutagenized populations, ecotypes, inbred lines and recombinant inbred line populations. These populations can be used for analyses of gene function. URGV also developed TILLING and EcoTILLING platforms to identify mutations of targeted genes of pea, tomato (Carriero *et al*. 2004) and rapeseed and the facilities named PETILL, TOMATILL and RAPETILL, respectively, in order to fill the gap between structural and functional genomics.

Current status of EcoTILLING

EcoTILLING is a high throughput strategy for identifying and analysing single nucleotide variations in natural populations. The method was developed by Comai and colleagues (2004) for detecting multiple types of polymorphisms in natural populations. This technique is particularly useful for plants requiring a long time for reproduction and for crops propagated vegetatively where creation of mutagenized populations is very difficult (Slade and Knauf 2005). This technique has been adapted in diverse species including rice, maize, *Lotus*, poplar, barley, melon and brassica.

EcoTILLING is an attractive approach because it can be applied to any organism regardless of its genome size, homogeneity and ploidy levels. This technique can be used in a high throughput fashion for detection of polymorphisms of a specific gene or gene region of interest, or genome-wide variation between genotypes, or among germplasm and wild relatives. SNPs discovered in natural populations can be used as genetic markers for genetic mapping, association analysis, mutational profiling and determination of evolutionary trends. SNPs in structural and regulatory genes may play an important role in phenotypic variation.

The protocol of EcoTILLING is slightly different from TILLING as it uses a mixture of the genomic DNA from a queried individual and a reference individual. SNPs discovery for indexing variation is essentially the same as that used in TILLING (**Fig. 1**).

Arabidopsis

EcoTILLING was first applied to Arabidopsis by Comai *et al*. (2004). They used EcoTILLING to examine variations in five different genes, DNA methyl transferase 2 (*DNMT2*), DNA methyl transferase 1-2 (*DRMI 1-2*), a putative GATA-type transcriptor factor (*C7*), phytochrome interacting factor 2-4 (*P1F2-4*) and Exonuclease domain of Werner syndrome (*AtWERNERexo*) in 96 ecotypes and detected multiple types of polymorphism. They discovered 55 haplotypes in five loci, demonstrating that this method is very useful for discovering or surveying variations in many individuals. The most variable haplotype was identified in the *PIF2* locus. The function of the *PIF2* encoded protein is unknown. The haplotype variations are largely limited to intronic regions and most of them do not encode variations in protein (Comai *et al*. 2004). The Eco-TILLING method has been applied to 192 natural accessions of *A. thaliana* to identify desired natural allele of variants (Brady and Provart 2007).

In addition to single nucleotide polymorphism, the Eco TILLING detected variation in haplotypes included small insertions, deletions and microsatellite polymorphisms.

The approach can also be applied in polyploid and heterozygous organisms where single or multiple polymorphisms at two more alleles may exist.

Barley

In barley *mlo* and *Mla* genes are involved in the defense against powdery mildew. The *mlo*, a single copy gene, encodes a protein, which could be involved in the cell wall repair process. The *Mla* gene region contains multiple loci and is associated with plant defence. EcoTILLING techniques were used successfully as a tool to detect allelic variation and SNP markers for *mlo* and *Mla* by Mejlhede *et al*. (2006). A total of 11 *mlo* mutants and 25 natural variants were tested for *Mla* alleles, respectively. The identification of *Mla* allelic variation was somewhat complex due to the presence of highly similar paralogues. Among 11 mutants of the *mlo* gene either single point mutations or several base pair deletions were detected in the exon region. The *mlo* gene polymorphisms were detected between genomes when wild type was compared with *mlo* mutants, whereas gene polymorphisms were discovered in *Mla* within the genome for each barley variant.

Melon

Resistance to several RNA plant viruses is mediated by a translation initiator factor of the 4E and 4G families of proteins in diverse crops. A single point mutation was recognized in 4E (*elF4E*) which controls resistance to melon necrotic spot virus (MNSV) (Nieto *et al*. 2007). They utilized EcoTILLING in 113 different melon accessions and discovered a highly conserved exonic region of *elF4E* with six polymorphic sites. Sequencing of six surrounding regions indicated that all of them were silent nucleotide changes except one with non-silent nucleotide change correlated with MNSV resistance. Functional analysis of new alleles indicated that the new allele of *elFAE* might be accountable for MNSV resistance. The Ecotilling technique effectively identified a new source of resistance to plant virus from the

available accessions.

Poplar

Poplar (*Populous trichocarpa*) is a model woody plant that is an important plantation species for pulp and paper industries. It is a renewable energy resource and also has been used for phyto-remediation (Gilchrist *et al*. 2006). Gilchrist *et al*. (2006) used EcoTILLING for the first time as an SNP discovery tool in a genetically heterogeneous, long-lived dioecious species to identify variations. They used nine different genes (*poptrTBI*, *poptrLFY*, poptrMP, *poptrFSH*, *poptrCH1*, *poptr4CL3*, *poptrNPR1*, *poptrMPK4* and *poptr KNATI*) among 41 different populations. A total of 63 *P. trichocarpa* SNPs in 8191bps were identified from all of the nine candidate genes. The least number of SNPs were identified in *poptrTBI* which had only one SNP, while *poptrLFY* had 23 SNPs in a 1000bp region.

This study demonstrated that EcoTILLING can be used to identify specific SNPs which are invaluable for genotyping or hybrid identification. Once a variation or polymorphism is identified, the representative allele can be sequenced to identify the nucleotide change relative to the standard genotype.

Rice

Raghavan *et al*. (2007) demonstrated that EcoTILLING can be used for mapping specific genes in rice. Two oxalate oxidase loci were tested for detecting SNPs between the parental lines SHZ-2 and LTH, and their 215 recombinant inbred lines (RIL). They detected the presence of SNPs and confirmed genetic linkage of the loci with disease resistance using SNP marker and trait data. Raghavan *et al*. (2007) also detected the presence of SNPs in the *MYB1* locus between Azucena (Japonica) and SHZ-2 (Indica) rice and assayed this locus up to the BC10 generation, demonstrating that this technique could be applied to a backcross program to track the target allele.

Raghavan *et al*. (2007) also compared the EcoTILLING detection method between the LI-COR genotyping system and an agarose gel system using 48 genotypes and controls to detect SNPs in order to develop a cost effective method for genotyping. They found that it was possible to detect SNPs with agarose gel, although its sensitivity for amplicon detection was very low as compared to the LI-COR DNA analyzer (Raghavan *et al*. 2007).

EcoTILLING has been shown to have excellent potential for gene and/or QTL mapping in near isogenic lines and backcross inbred lines, and for marker-assisted back-crossing. With the availability of whole genome sequences in many crops, SNPs detection offers an alternative cost effective way to track specific genes in breeding pedigrees or segregating populations rather than sequencing. The SNPs discovery technique provides a valuable connection for evaluating orthologous markers across species.

Leung *et al*. (2004) targeted several candidate genes for drought tolerance for initial EcoTILLING in rice. The coding sequences of candidate genes, including *Pp2a4* (Protein phosphatase gene), *DREB1* (Drought Responsive Element Binding protein1), *TPP* (Trehalose 6-Phosphate Phosphatase), were surveyed using cultivated and wild germplasm. Mismatches were detected in these genes across a range of germplasm. Among them three putative SNPs were detected for *Pp2a4* out of 23 accessions, including Nipponbare, and three putative SNP haplotypes identified within 96 accessions for *DREB1*. In addition a distinct haplotype occurred for *TPP* out of 48 lines derived from a cross between IR64 and Kun Min Tsieh Hunan.

Sugarcane

Allelic diversity of sucrose phosphate synthase (*SPS*) genes and the role of the *SPS* gene in sugar accumulation were identified in the complex polyploid vegetatively propagated crop sugarcane using the EcoTILLING approach (McIntyre *et al*. 2006). A total of 227 progenies derived from a cross between a low sugar containing wild hardy accession, IJ76-514, and a high sugar producing commercial cultivar, Q165A were used for identification of markers affecting sugar content in Q165A. Ten SNPs were detected within a small 417 nucleotide bp region of *SPS* gene family III demonstrating the usefulness of SNPs for identification of alleles in sugarcane. Each SNP was assessed for its potential as a single dose marker. Only two SNPs, SNP231 and SNP322, were found to segregate as a single dose marker. These two SNPs were only present in two sequence haplotypes of Q165A. They were mapped near QTLs for increased sucrose content in two different homologous linkage groups. However, the two SNPs of this *SPS* gene family III were not associated with variation of sugar content (McIntyre *et al*. 2006).

Other plants

Sulfonylurea (SU) is the most commonly used herbicide in Japan to control weeds in rice fields. Wang *et al*. (2007) successfully used the EcoTILLING technique for rapid detection of point mutations targeting acetolactate synthase (*ALS*) genes of the SU resistant paddy weed, *Monochoria vaginalis*. They identified single nucleotide mutations in the codon CCT to CAT and another CCT to TCT. The two mutations resulted in disruption of the conserved amino acid Pro197 to His and Ser residues, respectively, which resulted in herbicide insensitivity. The herbicide insensitivity is caused by a single nucleotide mutation in a conserved region of the *ALS* gene (Wang *et al*. 2007).

Monde *et al*. (2007) used EcoTILLING in maize to compare three genes between B73 and 24 maize diversity lines and identified natural polymorphism within exon regions. With the EcoTILLING technique they also detected different levels of diversity within genes.

APPLICATION OF TILLING AND ECOTILLING IN ORNAMENTALS

In this review successful examples of using TILLING and EcoTILLING to identify gene functions and SNP markers in many plants have been reviewed. Over the last few years extensive genomic research has been conducted on model plants and various food crops to develop high throughput technologies. However, very little or almost no genomic research has been accomplished for ornamentals. The availability of whole genome sequences of *A. thaliana* and rice may allow simultaneous identification of functions for thousands of genes and their resultant phenotypes. In addition, the integration of molecular approaches with traditional approaches enables breeders to improve existing crops or develop novel crops. In most cases the results could be applied to ornamental plants (van Harten 2002).

Ornamental plants are typically grown in gardens, yards, parks, and homes to decorate outdoor and indoor environments. These include various groups including flowers, cut flowers and potted plants, tuber and bulb crops, foliage, annual and perennial shrubs and trees. Desired traits in ornamentals are flower colour, shape, longevity, vase life and morphology, flowering time, plant structure, type and variegation of leaves, pigmentation, fragrance and growth habit such as compact, or semi-dwarf.

The improvement of ornamentals requires genetic variation. Desirable natural variation, however, is often inadequate which restricts improvement of ornamentals. Because occurrence of spontaneous mutations is extremely low, the availability of desirable gene pools is limited. Mutation breeding has become a major and popular breeding tool for breeding ornamental plants. Induction of mutations to broaden natural genetic resources is very effective in adding new genetic characteristics to the existing gene pools. This could increase the prospects for genetic enhancement and creation of novel traits for existing ornamentals. Induced

mutations have been effectively applied in developing improved or new cultivars for both seed and vegetatively propagated ornamentals (Jain 2006). This approach, coupled with high throughput technologies, offers an unprecedented opportunity to generate completely novel and improved phenotypes of ornamentals.

Flower colour is one of the most important traits in ornamental plants. The qualitative and quantitative inheritance of flower colour components which can be observed visually is of specific interest to ornamental breeding. Visual observation, however, does not give dependable information as plants with the same flower colour can have very different petal colour components (Sparnaaij 1976).

Induction of mutations for physiological traits such as changes in photoperiodic reaction, tolerance against abiotic (low light and cold) stresses, increased resistance to diseases and insects, early or late flowering, and alteration of fragrance, shelf life or extended flowering duration etc. are much more difficult, costly and time consuming (van Harten 2002; Schum 2003). Selection of desired mutants for morphological traits is much easier than selection of desired physiological traits, which requires advanced and sophisticated approaches (Schum 2003). One of the major problems in ornamentals is limited knowledge about the molecular basis of quantitative traits. Recent progresses on whole genome sequencing make the understanding of gene functions on a large scale possible for model and non-model crops which opens up innovative channels for improving ornamentals. The high throughput reverse genetic approaches, TILLING and EcoTILLING, can be applied to any organisms to detect SNPs for specific traits. These technologies will allow the identification of trait specific markers for mutants, parental lines and available germplasm (Jain 2006).

Flowering time genes, flower and meristem identity genes, and flower pigmentation genes are essential for initiation and development of flowers. Genetic organ identity and developmental genes controlling floral variation and flowering time are an indication of adaptation to different environments (Alonso-Blanco *et al*. 2005). The function of flowering time genes has been studied from artificially induced mutants in Arabidopsis. Fourteen QTLs were found to contribute to naturally occurring flowering variations and six of the isolated loci have different functions, such as *FRI* and *FLC* control vernalization; *EDI*, *phyD* and *FLW1* are involved in photoperiod response, while *HUA2* affects both vernalization and photoperiod (Alonso-Blanco *et al*. 2005). Mutations discovered in flowering time genes of various plants in different loci include *EDI*, *HUA2*, *PHYA* in Arabidopsis, *Hd6* and *EhD1* in rice, *VRn2* in Einkorn wheat, and *Dwarf8* in maize. Mutations also have been identified in plant architecture traits in bread wheat loci *RhtB1*, *RhtD1*, maize locus *TB1* and inflorescence trait morphology in Arabidopsis locus *CAL*. These mutations are either missense or nonsense substitutions which resulted in allelic dysfunction by truncation of protein or mis-expression of altered protein (Alonso-Blanco *et al*. 2005).

The phytohormone ethylene plays a critical role in the initiation and regulation of senescence. Identification and isolation of relevant genes involved in ethylene production and action opens the way to improve flower longevity. A major breakthrough in ethylene biosynthesis was the establishment of S-adenosylmethionine (SAdoMet), as the precursor for ethylene biosynthesis. The first committed step of ethylene biosynthesis is the conversion of SAdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and finally ACC is oxidized by ACC oxidase to form ethylene. Molecular cloning of the ACC synthase (*ACS*) and ACC oxidase (*ACO*) genes led to the demonstration that these enzymes belong to a multigene family (Wang *et al*. 2002).

Mutagenesis technology will allow the development of allelic series for important traits. TILLING and EcoTILLING technologies have potential to discover missense mutations in ethylene biosynthetic genes in order to improve flower longevity by reducing ethylene reactivity i.e., by inactivating either *ACS* or *ACO* gene, or by interrupting other steps in the ethylene biosynthetic pathway.

Understanding the molecular basis of flower form and its colour variation is necessary for integrating new flower colour and forms, which is an essential part of developing new ornamental varieties. The key pigments responsible for plant colour are the flavonoids, carotenoids, chlorophylls and betalains (Davies *et al*. 2003). Anthocyanins, including anthocyanidins, perlargonidin, cyaniding and delphinidin are the most common flavonoids influencing colours. The production of anthocyanins in a particular tissue depends on the activity of the genes involved in biosynthetic pathways. Modification of colour can be performed by inhibiting or preventing or modifying the key flavonoid biosynthetic enzyme chalcone synthase (CHS) (Davies *et al*. 2003) and other enzymes involved. With induction mutation, appropriate regulatory genes in the biosynthetic pathway can be modified to alter flavonoid profiles. This would generate a range of interesting, novel, and attractive phenotypic ornamentals which will have commercial value.

The change in phenotype might result from the absence of a specific biosynthetic enzyme. Use of a combination of traditional mutagenesis and high throughput TILLING and EcoTILLING could be an attractive approach to mutate specific genes for the generation of novel phenotypes for particular ornamental lines. By and large, both TILLING and EcoTILLING have a unique prospect and exciting future in elucidating the genetic or genomic basis of qualitative and/or quantitative traits, such as resistance to biotic stress, abiotic stress, longevity, enhanced fragrance, wide adaptability, short growing season, superior blooming, and traits affecting ornamental plant appearance.

PERSPECTIVES FOR THE FUTURE

One of the major tasks of forward genetics is to associate phenotypes with the corresponding mutated genes and one of the best ways to definitively identify a function for a gene is to find out what happens when it is inactivated leading to the concept of reverse genetics. Reverse genetics starts with genes, and aims at mutating them to study the resulting phenotype. The two disciplines are separated by many decades but complementary to each other today. Progress in molecular biology allowed the identification of physical genes, and to translate this "genetic logic" into a molecular picture. But reverse genetics requires having a means to selectively mutate a chosen gene. In yeast and mammals the inactivation of the chosen genes was commonly accomplished by homologous gene targeting (Scherer and Davis 1979) and the RNAi technique (Fire *et al*. 1998). In plants, the most common methods for producing reduction of function mutations are antisense RNA suppression (Finnegan *et al*. 1996), insertional mutagenesis (Alonso *et al*. 2003), and transposon mutagenesis (Tissier *et al*. 1999). However, antisense RNA suppression requires considerable effort for any given target gene before knowing whether it will work (McCallum *et al*. 2000a, 2000b), and insertional mutagenesis and transposon mutagenesis occur at a low frequency per genome and a substantial fraction of genes will be missed owning to inadequate sampling or insertion site bias (Henikoff and Comai 2003). As a result, general and efficient reverse genetic methods are needed to obtain knockout mutations.

The advent of TILLING and EcoTILLING technologies has provided a powerful tool to scientists for carrying out reverse genetics research for any plants because TILLING can provide allelic series of mutations, including knockouts. In addition, TILLING can be applied even if genomic sequencing is limited to selected target genes. The high density of chemically induced point mutations makes TILLING suitable for targeting small genes, and it allows a researcher to focus on single protein domains when targeting larger genes (Henikoff and Comai 2003). In contrast to antisense RNA techniques and insertional mutagenesis, TILLING is completely general, as chemical mutagenesis has been suc-

cessfully applied to most plant species. EcoTILLING, which is an expansion of the TILLING technology for identification of genetic variations in natural populations, is broadly applicable to any plants including ornamentals. As there are no genomic sequencing efforts ongoing in ornamentals, the application of TILLING to ornamental breeding would help breeders to create new plant types with desirable traits for decoration and the application of EcoTILLING would help researchers to understand ornamental crops at the molecular genetics level for genetic manipulation and enhancement. With the expansion of genomic sequence information in model and crop plants, numerous new candidate genes are expected to be identified from genome-wide expression studies or from comparative analysis across species. However, the paucity of polymorphism often limits the use of these genes in association or mapping analyses. Provided that enough sequence information is available to obtain PCR-amplicons, mismatch detection offers a simple way to track specific genes in breeding pedigrees and segregating populations without upfront investment in sequencing. The ease of detecting SNPs in related genetic materials by TILLING and EcoTILLING implies that many more candidate genes can now be used for mapping, pedigree analyses and for marker assisted breeding. Because TILLING and EcoTILLING can use pooled DNA samples to increase throughput and DNA polymorphisms can be detected by non sophisticated instrumentation or costly labeled primers (Raghavan *et al*. 2007), they become more efficient and affordable to any laboratories, particularly in developing countries.

ACKNOWLEDGEMENTS

We thank Agriculture and Agri-Food Canada for its financial support to the authors' laboratories.

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