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Progress in TILLING as a tool for functional genomics and improvement of crops

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Abstract Food security is a global concern and substantial yield increases in crops are required to feed the growing world population. Mutagenesis is an important tool in crop improvement and is free of the regulatory restrictions imposed on genetically modified organisms. Targeting Induced Local Lesions in Genomes (TILLING), which combines traditional chemical mutagenesis with high-throughput genome-wide screening for point mutations in desired genes, offers a powerful way to create novel mutant alleles for both functional genomics and improvement of crops. TILLING is generally applicable to genomes whether small or large, diploid or even allohexaploid, and shows great potential to address the major challenge of linking sequence information to the function of genes and to modulate key traits for plant breeding. TILLING has been successfully applied in many crop species and recent progress in TILLING is summarized below, especially on the developments in mutation detection technology, application of TILLING in gene functional studies and crop breeding. The potential of TILLING/EcoTILLING for functional genetics and crop improvement is also discussed. Furthermore, a small-scale forward strategy including backcross and selfing was conducted to release the potential mutant phenotypes masked in M_2 (or M_3) plants.

Keywords: Crop breeding; functional genomics; mutation detection; TILLING
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INTRODUCTION

With the world's population forecasted to reach 9 billion by 2050, food security has become a critical global challenge for the twenty-first century. It has been estimated that cereal production needs to increase by 50% by 2030 (Allen et al. 2011) and 70% by 2050 to adequately feed the huge population (FAO 2009). To meet this target, a 37% increase in the historical annual incremental rate for crop production is required (Tester and Langridge 2010), which shows clearly that the scope of the envisaged increases is unprecedented (Mba 2013). However, the extremely narrow genetic base of the available crop varieties and the parental lines for creating new varieties nullify efforts to enhance productivities and increase vulnerabilities, which thereby threaten food security. It is therefore necessary to investigate wider sources of heritable variations for crop improvement. In addition to the application of novel genetic resources including landraces and wild relatives in crop improvement, the use of mutagenesis is an alternative method for developing new alleles of genes that control the traits desired for superior crop varieties in the twenty-first century (Sikora et al. 2011; Mba 2013). Over the years, mutagenesis has generated a large amount of genetic variability and has played significant roles in crop improvement throughout the world. Records maintained by the Joint FAO/IAEA Programme show that over 3,200 crop varieties with one or more useful traits obtained from induced mutations were released officially or commercially worldwide during the last 40 years (FAO-IAEA, Mutant Variety Database (MVD), 2013, http://mvgs.iaea.org/AboutMutantVarieties.aspx). Historically, the use of mutagenesis in breeding has involved forward genetic screens where the selected individual mutants with improved traits were adopted into breeding projects. However, this approach has its limitations though it has clearly proved very successful. For example, it is difficult to identify a single individual with novel phenotypes within a large population, and many mutations have no detectable effect on phenotype due to genetic redundancy caused by gene duplication or polyploidy. Recently, reverse-genetic approaches have permitted the silencing or interruption of individual target genes, providing the opportunity to link sequence variation information to traits and to investigate gene function (Parry et al. 2009; Gilchrist and Haughn 2010).

In this century, there has been a dramatic increase in the amount of genome sequence data available for major crop species, which subsequently encouraged the development of reverse-genetics approaches. These approaches rely on the

disruption of candidate genes by mutagenesis, transposons, T-DNA insertion or RNA interference (RNAi), which allow progress to be made on the major challenge of linking sequence information to gene function and on determining their contribution to important traits (Parry et al. 2009). However, these techniques have disadvantages: RNAi, which is based on post-transcriptional gene silencing, has variable success rates and relies on time-consuming vector construction and plant transformation (Fu et al. 2007). T-DNA or transposon insertional mutagenesis is also dependent on efficient plant transformation, which is only available in some crops, notably maize, and now rice (Jeon et al. 2000; Kolesnik et al. 2004), but has not been extended to wheat. In any case, these insertional approaches are likely to result in complete null mutants of the target gene rather than in generating allelic series of mutations with partial loss-of-function, which are favorable for gene functional analysis but may be less useful for direct crop breeding. Additionally, the number of insertion lines required for full genome coverage may increase to unrealistic levels because the insertion loci within the genome are unlikely to be distributed randomly (Zhang et al. 2007).

Thus, Targeting Induced Local Lesions IN Genomes (TILLING), which combines traditional chemical mutagenesis (usually ethyl methanesulfonate, EMS or sodium azide) with high-throughput genome-wide screening for point mutations in desired genes, has been developed in response. This method may be preferable to other reverse-genetics approaches for various reasons. EMS generates a large spectrum of mutations, including missense and truncation mutations, allowing more flexibility than insertional mutagenesis or transgenesis (McCallum et al. 2000b). Furthermore, EMS can produce random point mutations at high frequency in polyploid plants. This allows multiple alleles of a specific gene to be obtained in a small population regardless of the genome size (Greene et al. 2003; Till et al. 2007; Sabetta et al. 2011). Thus, the TILLING method is aptly characterized as "Traditional Mutagenesis Meets Functional Genomics" and is useful for both functional genomics and crop improvement. Although the nucleotide sequence alone may provide enough information to infer something about a gene's function, such predictions must be validated phenotypically (Rothe 2010). TILLING can provide the empirical validation needed for this type of sequence-driven hypothesis (McCallum et al. 2000b). The importance of this locus-to-phenotype methodology is demonstrated by the success of the *Arabidopsis* TILLING Project (ATP) (Till et al. 2003b; Henikoff et al. 2004). TILLING also finds application in crop improvement, as the mutations identified by TILLING can be readily used in traditional breeding programs since it is non-transgenic and the novel variations are inherited stably (Dong et al. 2009a; Uauy et al. 2009; Kumar et al. 2013). In addition, TILLING screening can identify individuals with heterozygous recessive mutations, which are usually missed in phenotyping and the polymorphism of the target gene can be used in marker-assisted selection during cultivar development (Wilde et al. 2012). TILLING resources have been developed for several crop species such as wheat (Dong et al. 2009a; Chen et al. 2012; Rawat et al. 2012), rice (Wu et al. 2005; Till et al. 2007), maize (Till et al. 2004b), barley (Caldwell et al. 2004; Talame et al. 2008; Gottwald et al. 2009; Lababidi et al. 2009), soybean (Cooper et al. 2008), sorghum (Xin et al. 2008; Blomstedt et al. 2012), potato (Muth et al. 2008), peanut (Knoll et al. 2011), oat (Chawade et al. 2010), and tomato (Minoia et al. 2010; Piron et al. 2010) (also see Table 1). Consequently, the use of chemically induced mutagenesis has had a renaissance with the development of TILLING method. Moreover, TILLING technology has been improved through altering the mutation screening method using advanced molecular techniques (Dong et al. 2009a; Parry et al. 2009).

We review here the progress in the development and adaptation of TILLING in crop species, and focus more on recent advances in mutation screening methods, potential for functional genomics and crop improvement and also the prospect of TILLING in the future. Furthermore, a small-scale forward strategy is described for releasing the potential visible mutation phenotype that masked in M_2 plants.

MUTATION DETECTION BY TILLING

TILLING is a reverse-genetic method combining chemical mutagenesis with polymerase chain reaction (PCR)-based screening to identify point mutations in regions of interest, which was first reported in the late 1990s (McCallum et al. 2000a). The original TILLING method used a commercial denaturing high-performance liquid chromatography (DHPLC) apparatus for mutation discovery. However, the DHPLC method does not scale up easily for high throughput and alternative technologies are needed for improving TILLING efficiency. A method combining enzymatic digestion with the mismatch cleavage endonuclease CELI (endonuclease from celery) and gel electrophoresis with the LI-COR gel analyzer system was developed to provide a low cost, high-throughput platform for mutation discovery by TILLING (McCallum et al. 2000b; Colbert et al. 2001). TILLING progressed rapidly with the advent of mutation detection by CELI, which cleaves DNA at the 3′ side of the mismatch (Oleykowski et al. 1998; Colbert et al. 2001). The resulting cleaved PCR fragments, amplified with two different fluorescently labeled primers, are generally separated by denaturing polyacrylamide gel electrophoresis (denaturing PAGE) using the LI-COR DNA analyzer, then the precise base position of a mutation can be pinpointed (Till et al. 2003a, 2003b). The protocol published by the ATP has been widely used as the most efficient and cost-effective method (Till et al. 2003b). Even crude celery juice extracts (CJE) could be used successfully, making this protocol accessible to many laboratories (Till et al. 2004a). Other enzymes have also been successfully used to cleave mismatches, for example, SURVEYOR/CEL II or ENDO I endonuclease (Triques et al. 2008; Voskarides and Deltas 2009; Okabe et al. 2013).

To further improve the throughput and the efficiency of mutation detection of TILLING, a quite diverse set of old and new technologies was reported as alternatives in different plant species (Wang et al. 2012). These alternative detection methods include those based on electrophoresis of CELI digested products, such as non-denaturing polyacrylamide gel, agarose gel electrophoresis, and microchip electrophoresis system, and those that do not rely on mismatch cleavage by endonucleases, such as high-resolution melting (HRM) analysis and next-generation sequencing (NGS).

The detection system with non-denaturing polyacrylamide gel or agarose gel electrophoresis for TILLING does not rely on the use of high-throughput electrophoresis equipment, such as

Table 1. Description of TILLING resources developed in model and crop plants

Species	Ploidy	Mutagen	M_2 size	Mutation frequency (1/kb)	Traits	Country	Mutation detection technology	References
Rice	2x	EMS	768	1/294	–	USA	LI-COR	Till et al. 2007
Rice	2x	Az-MNU	768	1/265	–	USA	LI-COR	Till et al. 2007
Rice	2x	MNU	767	1/135	Leaf emergence	Japan	CE	Suzuki et al. 2008
Rice	2x	Gamma rays	2,130	1/6190	–	Japan	Agarose gel	Sato et al. 2006
Rice	2 x	DEB EMS	–	1/1000	–	Philippines	LI-COR	Wu et al. 2005
Wheat	2x	EMS	1,400	1/1300	Grain quality and lignin biosynthesis	USA	Agarose gel	Rothe 2010
Wheat	2x	EMS	1,532	1/92	Waxy and lignin	USA	Agarose gel	Rawat et al. 2012
Maize	2x	EMS	750	1/485	Chromomethylase	USA	LI-COR	Till et al. 2004a, 2004b
Barley	2x	EMS	9,216	1/1000	Floral organ regulation	UK	dHPLC	Caldwell et al. 2004
Barley	2x	EMS	10,279	1/500	Row type morphology and immunity to fungus	Germany	LI-COR	Gottwald et al. 2009
Barley	2x	NaN3	3,148	1/374	Virus resistance and immunity to fungus	Italy	LI-COR	Talame et al. 2008
Sorghum	2x	EMS	1,600	1/526	Forage digestibility	USA	LI-COR	Xin et al. 2008
Brachypodium distachyon	2x	NaN_3	5,530	1/396	Lignin biosynthesis	France	LI-COR	Dalmais et al. 2013
Tomato	2x	EMS	1,926; 4,741	1/574; 1/322	Shelf life	Italy	LI-COR	Minoia et al. 2010
Tomato	2x	EMS	8,225	1/737	Proline biosynthesis	The Netherlands	CE, HRM	Gady et al. 2009
Tomato	2x	EMS/neutron	15,000	–	Virus resistance	Israel	NGS	Menda et al. 2004, Rigola et al. 2009
Tomato	2x	EMS	4,759	1/574	Virus resistance	France	LI-COR	Piron et al. 2010
Tomato	2x	EMS	3,052	1/1237	Shelf life	Japan	LI-COR	Okabe et al. 2011, 2012
Pea	2x	EMS	8,000	1/669	Gibberellin metabolism	France	LI-COR	Triques et al. 2007
Sunflower	2x	EMS	3,651	1/475	Fatty acid biosynthetic pathway and downy mildew resistance	Italy	LI-COR	Sabetta et al. 2011
Brassica rapa	2x	EMS	9,216	1/60	DNA methylation	UK	CE (ABI3730)	Stephenson et al. 2010
Brassica oleracea	2x	EMS	8,750	1/447	Wax biosynthesis and dwarf stature	USA	LI-COR	Himelblau et al. 2009
Melon	2x	EMS	4,023	1/573	Fruit quality	France	LI-COR	Dahmani-Mardas et al. 2010
Melon	2x	EMS	2,368	1/1500	Disease resistance, fruit quality	Spain	LI-COR	Gonzalez et al. 2011
Lotus japonicus	2x	EMS	4,904	1/502	Nodule development	UK	LI-COR, CE	Perry et al. 2003
Arabidopsis	2x	EMS	3,072	1/300	–	USA	LI-COR	Greene et al. 2003
Arabidopsis	2x	EMS	6,912	1/170	–	USA	LI-COR	Till et al. 2003a, 2003b
Wheat	4x	EMS	8,000	1/40	Starch quality	USA	LI-COR	Slade et al. 2005

(Continued)

Table 1. (Continued)

Species	Ploidy	Mutagen	M_2 size	Mutation frequency (1/kb)	Traits	Country	Mutation detection technology	References
Wheat	4x	EMS	1,368	1/51	Starch quality	USA	PAGE, LI-COR	Uauy et al. 2009
Soybean	4x	NMU	768	1/140	–	USA	LI-COR	Cooper et al. 2008
Soybean	4x	EMS	768; 529	1/550; 1/140	–	USA	LI-COR	Cooper et al. 2008
Rapeseed	4x	EMS	2,604; 7,110	1/130; 1/41	Oil quality	China	LI-COR	Wang et al. 2008b
Potato	4x	EMS	2,748	1/91	Starch quality	German	Direct sequencing	Muth et al. 2008
Peanut	4x	EMS	3,420	1/967	Seed quality	USA	LI-COR	Knoll et al. 2011
Tef (*Eragrostis tef*	4x	EMS	2,121	1/115; 1/370	Plant height	USA	NGS (Roche 454)	Zhu et al. 2012
Wheat	6x	EMS	10,000	1/24	Starch quality	USA	LI-COR	Slade et al. 2005
Wheat	6x	EMS	1,536	1/38	Starch quality	USA	PAGE, LI-COR	Uauy et al. 2009
Wheat	6x	EMS	2,348	1/37; 1/23	Starch quality and grain hardness	Australia	Agarose gel	Dong et al. 2009a,b
Wheat	6x	EMS	2,610	1/34; 1/47	Spike development	China	Agarose gel, PAGE	Chen et al. 2012
Wheat	6x	HII	4,500	1/84	Disease resistance	Australia	A probe method	Fitzgerald et al. 2010
Wheat	6x	EMS	4,244	–	Starch biosynthesis	Italy	LI-COR	Sestili et al. 2009
Wheat	6x	EMS	630	1/13	Grain hardness	USA	Direct sequencing	Feiz et al. 2009
Oat	6x	EMS	2,600	1/40; 1/20	Increased digestibility and improved food quality	Sweden	MALDI-TOF	Chawade et al. 2010

EMS, ethyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea; NMU, *N*-nitroso-*N*-methylurea; CE, capillary electrophoresis; HRM, high-resolution melting; HII, heavy ion irradiation; NGS, next-generation sequencing technology; PAGE, polyacrylamide gel electrophoresis.

the LI-COR DNA analyzer or ABI genetic analyzer, which use fluorescent end-labeled primers and are relatively expensive for individual laboratories. These have been successfully applied to detect EMS-induced mutations in large libraries (Till et al. 2003a; Slade et al. 2005; Suzuki et al. 2008; Dong et al. 2009a; Uauy et al. 2009; Chen et al. 2012). Briefly, the procedure of these non-fluorescence methods is similar to that of the LI-COR system, except that fluorescence-labeled gene-specific primers are not used. A detailed comparison between non-denaturing polyacrylamide gel and LI-COR gel (Uauy et al. 2009), agarose gel and LI-COR gel (Raghavan et al. 2006), and a modified agarose TILLING system (Dong et al. 2009a) were reported. We had verified the effectiveness of either non-denaturing polyacrylamide gel or agarose gel based TILLING/EcoTILLING system in screening single nucleotide polymorphisms (SNPs) in wheat (Figure 1) (Chen et al. 2011, 2012). A distinctive advantage retained by these non-fluorescence approaches was that SNPs in gene fragments with a length over 2 kb could be detected with high sensitivity in a short time (1–2 h) while for the LI-COR gel system, the suitable amplicon size was between 1.2 and 1.5 kb and needs a long-time run (about 5 h) (Raghavan et al. 2006; Chen et al. 2011, 2012). Overall, the non-denaturing polyacrylamide or agarose system simplified the procedure by only using instruments available in any basic molecular biology laboratory, making the TILLING approach more accessible to a larger set of biologists and crop improvement programs.

The microchip electrophoresis system (MCE-202 MultiNA, Shimadzu, Kyoto-shi, Japan) is a new strategy for screening SNPs for TILLING (Chen et al. unpubl. data, 2012). Generally, the CELI digested products are run through capillary electrophoresis in which SNPs yield novel fragments in a virtual gel image and also show different peaks in a simulated electropherogram (Figure 2). In addition, microchip electrophoresis systems export a results table, showing the size (bp) and concentration (ng/μL) of different fragments, helping to find the positive samples. This microchip electrophoresis system has a promising future with the flowing advantages: easy use; equipped with automatic analysis functions (MultiNA Viewer software); high throughput (automated high-speed electrophoresis separation); high-precision size estimation and quantitation. For those laboratories dissatisfied with agarose or polyacrylamide gel electrophoretic methods of detection, this microchip-based capillary electrophoresis system would be a good choice for mutation scanning. However, this method has not been largely applied in TILLING programs and the system still needs to be optimized because the backgrounds of the simulated electropherograms are relatively strong, possibly caused by the random or excessive digestion of PCR products by CELI. Purification of digested products may be required before electrophoresis, but this was not verified in this study.

High-resolution melting analysis is one of the recent developed techniques for mutation detection that do not rely on mismatch cleavage by endonucleases (Zhou et al. 2004;

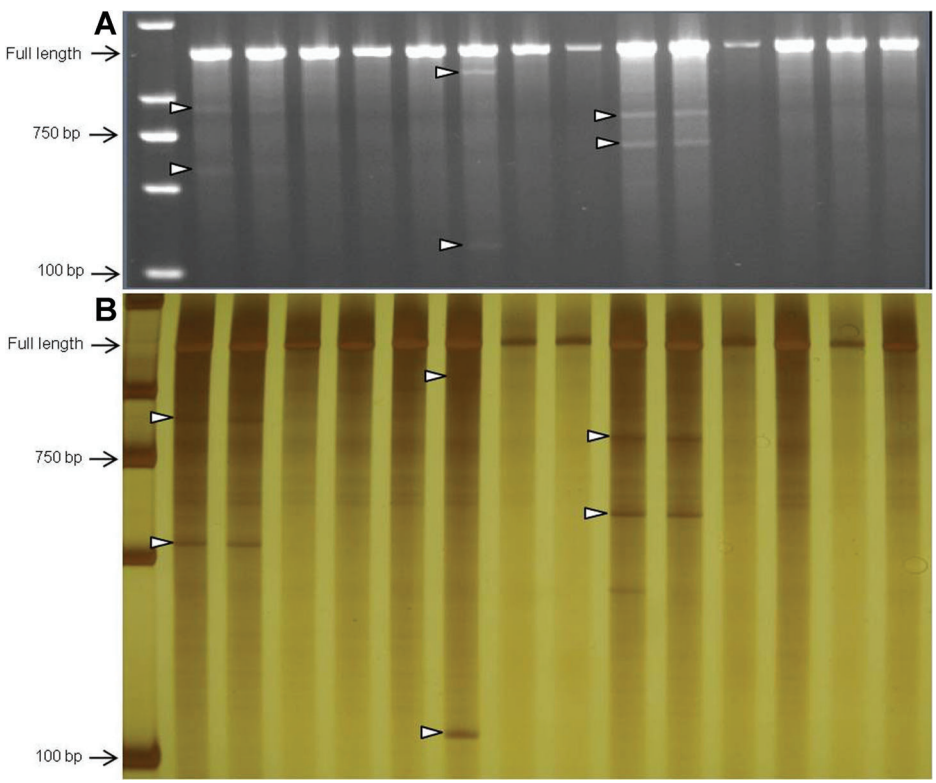
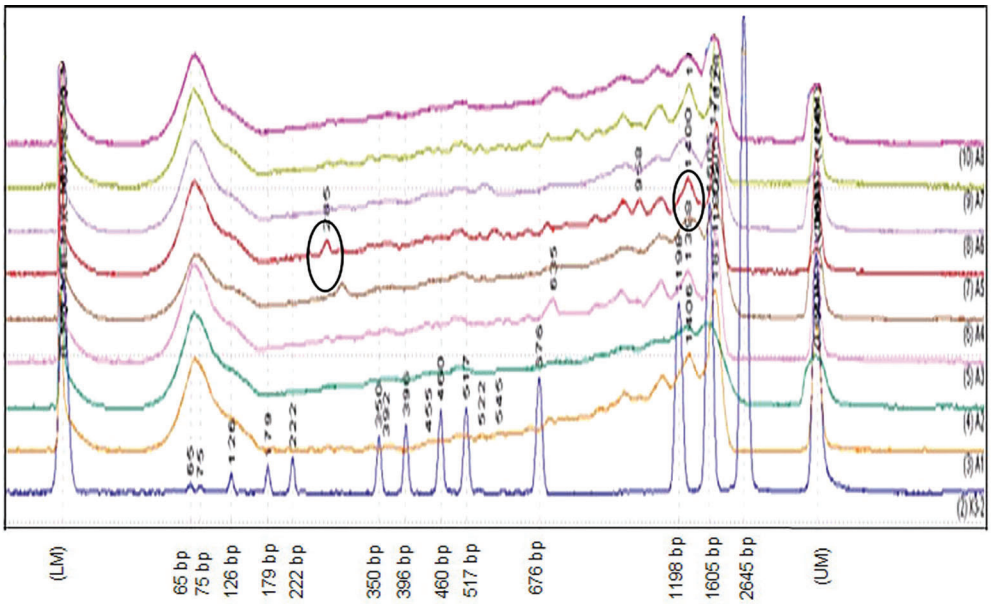


Figure 1. Digested bands detected with agarose gel (A) and non-denaturing polyacrylamide gel (B) electrophoresis
Putative mutations in the pools (1, 2, 3, 4, 5) are identified by the presence of two bands (indicated by white arrows), with sizes adding up to the full-length polymerase chain reaction (PCR) product (also see in Chen et al. 2012).



Results table

No.	Size (bp)	Conc. (ng/μL)
1	312	0.82
2	1464	1.75
3	1804	1.96

Figure 2. Digested bands detected with microchip electrophoresis system (MultiNA)
Putative mutations in the pool are identified by the presence of two peaks (indicated by black circles), with sizes adding up to the full-length polymerase chain reaction (PCR) product. The results table shows the size (bp) and concentration (ng/μL) of the two digested bands (No. 1 and No. 2) and the full-length fragment (No. 3) detected in the positive pool. LM and UM showed the ladder information. Further analysis is impossible to confirm the mutation.

Gady et al. 2009; Parry et al. 2009; Botticella et al. 2011). HRM depends on the loss of fluorescence from intercalating dyes bound to dsDNA during thermal denaturation (Ririe et al. 1997). These dyes, such as LCGreen/LCGreen Plus and CYTO9, have low toxicity to PCR and can therefore be used at high concentration to saturate the dsDNA PCR product. The combination of these characteristics provides greater melt sensitivity, stability and higher resolution melt profiles and makes it feasible to detect SNPs in PCR fragments, even in somatic mutations and methylations (Dong et al. 2009b; Li et al. 2010). Accurate control of temperature and continuous monitoring of fluorescence in instruments such as the Lightscanner (Idaho Technology, Salt Lake City, UT, USA) or the Rotor-Gene (Qiagen, Hilden, Germany) allows detection of single base mismatches in target fragments from the mutant pools. This method is especially suited for target genes with multiple small exons separated by large introns as the detection sensitivity of HRM is optimized with amplicons of <450 bp (Parry et al. 2009). Moreover, HRM is a rapid assay and completed in a closed tube, no digestion and gel separation steps are required, which makes it a good choice for screening mutations in TILLING or EcoTILLING. For hexaploid wheat, mutation screening by HRM requires two-step nested PCR amplifications, a larger fragment containing several coding regions was first amplified using genome-specific primers, then HRM analysis was conducted using primers specific for each exon or part thereof (Parry et al. 2009); an example is shown in Figure 3. For diploid plants, the procedure could be simplified as a one step PCR as there is no need to distinguish the different homoeologous copies (Lochlainn et al. 2011). As the melt analysis following PCR is extremely rapid, the throughput of this method is equal to or greater than that of the CELI based method and is, arguably, easier to establish. In addition, Dong et al. (2009b) reported that combining HRM scanning with sequence analysis using Mutation Surveyor is sensitive enough to detect a single nucleotide mutation simultaneously in the heterozygous state in a mixed PCR amplicon containing three homoeologous gene fragments of bread wheat.

Along with the costs of large-scale DNA sequencing fall dramatically, the application of NGS, which permits multiplexing of gene targets and genomes, ultimately leads TILLING to be an *in silico* procedure (Wang et al. 2012). NGS technologies (Roche 454, Illumina/Solexa, and ABI SOLiD) are used to discover mutations regardless of the choice of mutagen. It has also helped to bypass some challenges presented in the conventional TILLING strategies, as the pools could be as high as 40- to 50-fold on some NGS instruments with high throughput and at reasonable cost (Weil 2009). In addition, mutation discovery in polyploids may be more efficient using sequencing approach that data collected from a single starting molecule make it not necessary to target-specific homologs (Tadele et al. 2010). TILLING by sequencing had been demonstrated in an EMS-induced tomato population using the Roche 454 technology combined with a multi-dimensional pooling strategy and two mutations in the *eIF4E* gene were identified among more than 3,000 M_2 families in a single GS FLX sequencing run, and six haplotypes of the *eIF4E* gene was discovered by re-sequencing three amplicons in a subset of 92 tomato lines (Rigola et al. 2009).

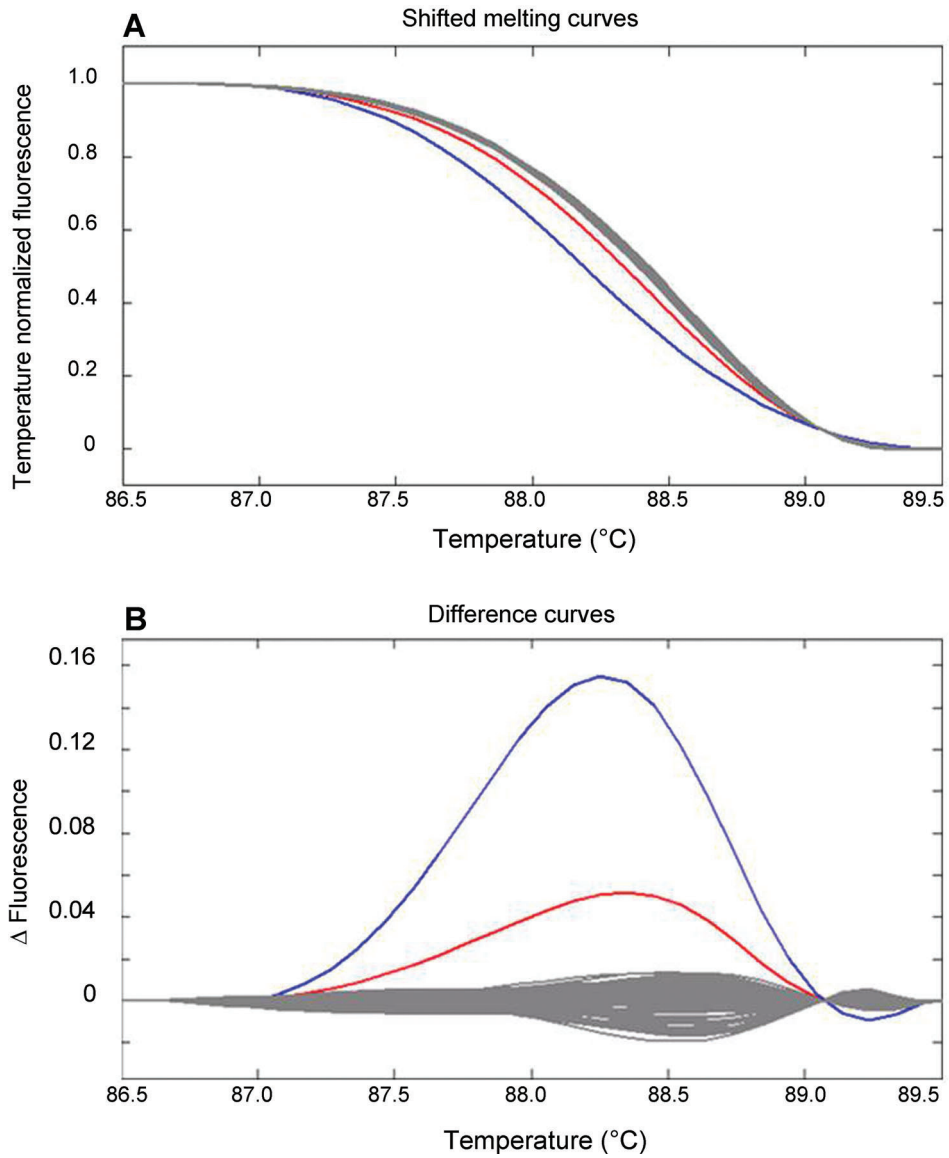


Figure 3. Mutation detection for dwarfing gene *Rht-D1* in bread wheat using high-resolution melting analysis (HRM)
The HRM data are presented as two different parts: **(A)** displays normalized melting curves for the tested amplicons, whereas **(B)** shows difference curves displaying the difference in fluorescence (ΔF) against temperature from melt analysis of the amplicons in relation to the wild-type in a 96-well plate (2× pools) from an ethyl methanesulfonate (EMS)-mutagenized population of bread wheat cv. Cadenza. Wild-type samples are in gray, the putative mutant pools identified through their different melt profiles are colored. These amplicons were sent for DNA sequencing to confirm the mutant site in the gene. In this project, the first-round homeolog-specific polymerase chain reaction (PCR) targeted the bread wheat *Rht-D1* gene; the second round amplicon was 200 bp of its first exon. LCGreen Plus dye was included in the second round PCR for melt detection and heteroduplex melt analysis was carried out on the Lightscanner (Idaho Technology) (AL Phillips et al. unpubl. data, 2012).

Actually, in the near-term, whole genome sequencing will remain expensive for the discovery of induced mutations in a highly mutagenized plant in a large population. However, 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 improved pooling methods. A simple multi-dimensional pooling strategy was demonstrated for rice and wheat by Illumina sequencing of target genes amplified using bar-coded primers for sample tracking from pooled DNA templates. After sequencing, mutations were identified by comparison with a reference sequence of wild-type, thus circumventing the requirement for detection by enzymatic cleavage or PCR-based melting techniques, which enabled the detection of an individual mutant and its molecular identity without the need for extra deconvolution of pools and more sequencing steps (Tsai et al. 2011). A probabilistic

method for calling TILLING-induced mutations from high-throughput sequencing data of overlapping DNA pools was tested on wheat and rice mutagenized populations and showed a high efficiency on discovering SNPs in large populations (Missirian et al. 2011). However, these techniques still rely on PCR amplification of individual gene of interest and remain a targeted approach. Should sequencing costs continue to decrease at their current rate, untargeted methods can be adopted. To date, several large-scale TILLING services including the Seattle TILLING Project, the Rice TILLING Project, and the Maize TILLING Project have evaluated NGS technologies for TILLING using the Solexa and ABI SOLiD platforms (http://genome.purdue.edu/maizetilling/; http://tilling.fhcrc.org/). Moreover, the UC Davis TILLINGCore has converted from LI-COR-CELI assays to the NGS of pooled genes using the Illumina GAII and Hi-Seq platform (http://tilling.ucdavis.edu/index.php/Main_Page) and provided a commercial workshop for scientists covering the experimental and bioinformatic components of TILLING by Sequencing. This platform was also used in the USA project "Efficient identification of induced mutations in crop species by ultra-high-throughput DNA sequencing" (Kurowska et al. 2011). The early successes of NGS in TILLING by large-scale facilities will be valuable for smaller groups, although the cost of the required equipment and reagents are still limiting its application.

Practically, every method has its distinctive value though they are really not applicable in any case. For example, the agarose gel system is most suitable for small labs with low-budgets while the HRM system is especially compatible with the groups whose work are hampered by CELI preparation or PCR product digestion. However, the LI-COR system is still the most widely used method for mutation screening in TILLING projects (Table 1). Additionally, Table 2 shows the instruments required, cost per sample, processing time, advantages and disadvantages of each method in PCR and mutation screening phase of TILLING, which provides information for finding the "best methodologies" for different TILLING groups.

While the development of new technologies is very exciting; however, it is important to note that the current state of the art for TILLING mutation discovery, enzymatic mismatch cleavage followed by gel detection, is not a bottleneck in terms of time or cost for the TILLING system. The major bottlenecks lie in combining mutations in different homoeologous genes of polyploid species and in subsequent mutant characterization. Developing rapid and low-cost phenotyping procedures is one of the most important ones for improving the efficiencies of TILLING. Advances in mutation discovery will, however, greatly benefit TILLING facilities that provide fee-based mutation discovery, but not phenotyping services.

The design of an amplicon is a crucial step for TILLING analysis. The selection of a suitable gene region provides a higher probability to identify nonsense or useful missense mutations by TILLING screens. This can be achieved with the software Codons Optimized to Discover Deleterious Lesions (CODDLE), a web-based sequence analysis tool to assist in TILLING process (http://www.proweb.org/coddle). CODDLE takes genome sequence data and a protein alignment as input, selects a fragment containing the highest density of potentially deleterious mutations caused by treatment with chemical mutagens, then runs the Primer 3 program to generate PCR primers to amplify the selected region. After mutations have been discovered by TILLING, they can be graphically displayed with the Project Aligned-Related Sequences and Evaluate SNPs (PARSESNP) program that incorporates the use of PSSM and SIFT to predict the effect of missense mutations on protein function (http://www.proweb.org/parsesnp/). Mutations with PSSM scores higher than 10 indicate that a missense change is more likely to have a damaging effect on the protein function. A mutation with an SIFT score lower than 0.05 is predicted to be deleterious. Another feature of PARSESNP is the ability to predict changes in the restriction enzyme recognition sites (Stemple 2004; Till et al. 2006) to allow design of simple Cleaved Amplified Polymorphic Sequences (CAPS) markers. Thus, bioinformatics tools used throughout the TILLING process from the beginning to the end improves the speed and efficiency of mutation detection.

TILLING IN FUNCTIONAL GENOMICS AND CROP IMPROVEMENT

What is unique for TILLING as a functional genomics strategy compared with transgenic approaches, such as T-DNA, transposon insertion and anti-sense or RNAi is the identification of numerous mutations within a target region of the genome without the need for genetic transformation. TILLING can also be applied to any species, regardless of its genome size and ploidy level (Jeon et al. 2000; Weigel et al. 2000; Alonso et al. 2003; Hsing et al. 2007; Parry et al. 2009; Kuntz 2012). Furthermore, its relatively easy operation and being less time consuming also make TILLING popular in gene functional studies and there have been many successful examples to date. For example, Javot et al. (2007) identified and characterized an EMS mutant allele of MtPT4 (*mtpt4-1*) by TILLING in *Medicago truncatula*, and confirmed that MtPT4 was essential for symbiotic Pi transport and development of arbuscular mycorrhizal symbiosis (Javot et al. 2007). Ronceret et al. (2009) identified the function of the conserved CR1 region of the Poor Homologous Synapsis 1 (PHS1) gene in maize through analyzing a point mutation named phs1-R148H by TILLING (Ronceret et al. 2009). To confirm the function of *E1* in delaying flowering of soybean, which was delimited through positional cloning, three independent *E1* missense mutants by TILLING within an EMS-treated soybean population were identified and used for genetic and phenotypic analysis to support their hypothesis (Xia et al. 2012). The *FEA2* locus controlling quantitative variation in maize kernel row number was identified through TILLING analysis of an allelic series of new *fea2* alleles with partial loss of *FEA2* function (Bommert et al. 2013).

TILLING in polyploids involves more effort on the downstream of mutation discovery, as the phenotype of a single homozygous mutant may be masked by the wild-type homoeolog(s) present in the other genome(s). In hexaploid wheat, for example, it is often necessary to identify mutations in the A, B, and D homoeologs and to combine these alleles by crossing and selfing over three generations to create a triple mutant that is more likely to display a phenotype. One consequence of this is that it is difficult to analyze allelic series of partial loss-of-function mutations in polyploid species as the number of combinations of alleles quickly becomes unmanageable, although it is theoretically possible to combine an allelic series in one homoeolog with null mutations in the other(s). Clearly, the high rate of mutation achievable in

Table 2. Different methods used in screening of TILLING mutation

Methods	Instruments	Processing time	Pooling depth	Cost per sample*	Advantages	Disadvantages
LI-COR gel analyzer system	Li-COR 4300	96 samples per run/5 h	Eightfold	0.13	High sensitivity; high throughput	Need expensive fluorescence-labeled primers and fluorescence analyzer; samples need purification before loading
Non-denaturing PAGE system	Vertical electrophoresis system and a camera	96 samples per run/3 h	Fourfold	0.10	Non-fluorescence; lower cost; more accessible	Sensitivity is relatively lower than fluorescence system
Agarose gel electrophoresis system	Horizontal electrophoresis system and a transilluminator	48 samples per run/1 h	Fourfold	0.07	Non-fluorescence; time-saving; lower cost; easy operation and more accessible	Sensitivity is relatively lower than fluorescence system
Microchip electrophoresis system	MultiNA	96 samples per run/3 h	Fourfold	0.08	High sensitivity; automated analysis	Need expensive electrophoresis equipment; with relatively strong background and needs to be optimized in the future
HRM analysis	Light scanner	96 samples per run/0.2 h	Twofold	0.15	Non-enzymatic screening system; high sensitivity; time-saving	The optimized amplicon is relatively short (<450 bp)
NGS	Roche 454 analyzer; Solexa analyzer; SOLiD analyzer	–	–	–	High throughput; time-saving; non-genome-specific primers can work; more efficient in polyploids	It remains a targeted approach and the cost is still high nowadays

*Costs in US Dollars estimated based on our lab at the recent price level of China and only include the reagents fee, and do not include the equipment costs. Cost per sample can also be reduced by improving the pooling depth.

polyploid species is more than offset by the need to identify null mutations in multiple homoeologs and to combine them by crossing. For these reasons, RNAi methods which, in principle, can target multiple genes simultaneously may be a more rapid approach to studying gene function than TILLING. Promisingly, random mutagenesis in TILLING can generate loss-of-function mutations with a relatively high probability (Slade et al. 2005; Dong et al. 2009a; Uauy et al. 2009), which provide the chance to combine mutations in different gene copies by crossing for functional genomics. Actually, TILLING, as well as other reverse genetics, can be applied simultaneously in the same gene for functional analysis (Hu et al. 2008).

The disadvantage of TILLING in gene functional studies is that only 5% of the total mutations in the EMS-mutagenized populations are truncations (Greene et al. 2003; Parry et al. 2009; Perry et al. 2009). Especially in diploid species, which have lower mutation frequencies (even relatively low levels of mutation can result in infertility), a very large population may be required to achieve saturated coverage of the genome (Greene et al. 2003; Caldwell et al. 2004). This indicates that a TILLING population of diploid plants where variations in all genes could be observed with a much larger population size, which requires much more advanced logistics in handling and maintenance. In contrast, insertion mutants produce non-functional alleles in most instances. However, insertional mutagenesis is often not saturated and thus requires an equally large population size to have a high probability of identifying an insertion in the target gene (Jeon et al. 2000; Zhang et al. 2007; Wang et al. 2012). This character makes it best suited for species with small genomes. To date, insertional mutagenesis is still not a valid alternative for many crop species. So, TILLING and other reverse-genetics methods complement each other in gene functional studies in various plant species.

The newly identified alleles in TILLING could be used for elucidating gene function, while it could also be used as valuable resources in crop improvement. Theoretically, the TILLING strategy could lead to the creation and identification of a series of new alleles for any gene of interest in any plant species, whereas most of the insertional methods are likely to result in the complete disruption of gene function, which will not produce the range of mutation strengths necessary for crop improvement. Additionally, the application of TILLING to crop improvement may help to compensate for the limited genetic variation in domesticated species. As we know, during domestication and subsequent selection, much of the genetic variations available in the wild crop progenitors have been lost (Gepts and Papa 2002). Thus, crop breeders have at times used landraces or wild relatives to introduce useful genetic variation (Graybosch 1998; Zamir 2001). As an alternative to the use of wild varieties, TILLING can be a way to introduce genetic variation in an elite germplasm without the need to obtain variation from exotic cultivars, thus avoiding introduction of undesirable agronomic traits. Moreover, the mutants identified by TILLING can be readily used in traditional breeding programs because it is non-transgenic and the novel variations can be inherited stably (Till et al. 2004b; Dong et al. 2009a; Slade et al. 2012).

TILLING focuses on first identifying mutations within genes of interest and then linking those mutations to a specific phenotype in plant breeding. To date, several groups have reported successes in crop improvement by the TILLING approach. Most noticeably in wheat, where over 200 alleles of the *waxy* gene that encode waxy enzymes ranging in activity from near wild type to null were identified through TILLING, which represent more genetic diversity than had been described in the preceding 25 years. Importantly, a line of bread wheat displaying a near-null waxy phenotype was generated using these mutations (Slade et al. 2005). Subsequently, durum and bread wheat lines with significantly increased amylose and resistant starch contents were developed using mutations of *SBEIIa* gene using a TILLING strategy (Slade et al. 2012). Moreover, in soybean where TILLING has proven useful in increasing the oleic acid content through the identification of mutations in the *RS2* and *FAD2-1A* genes (Dierking and Bilyeu 2009); in Sorghum where a trait associated with altered lignin content and increased digestibility was identified though TILLING screening of *COMT* (Xin et al. 2008); in oat, several different mutations in *AsPAL1* and *AsCslF6* genes (key genes in the lignin and β-glucan biosynthetic pathways) were demonstrated, which will be of great interest to oat breeders for creating new varieties with lower lignin content and higher β-glucan content (Chawade et al. 2010); in barley where phenotypic evaluation of the M_2 and M_3 generations showed a wide spectrum of morphological diversity that highlights the great potential of the barley TILLING resource for use in forward genetic screens while multiple alleles causing phenotypic changes of the two-rowed spike morphology were also obtained through TILLING detection of *HvHox1* gene (Gottwald et al. 2009); in tomato, two allelic mutants of *SlETR1* (ethylene receptor genes) that resulted in reduced ethylene responses were identified in a tomato TILLING library, which proved could be valuable breeding materials for improving the shelf life of tomato fruit (Okabe et al. 2011, 2012, 2013); in potato, a loss-of-function allele ($waxy_{E1100}$) that caused mis-splicing and protein truncation was identified and had been used to establish elite cultivars lacking granule-bound starch synthase I protein activity and producing high-amylopectin starch, suggesting TILLING could also be an efficient tool for exploring genetic variations of important agronomic traits in potato, a genetically complex and vegetatively propagated crop (Muth et al. 2008).

In a mutant population, some mutant phenotypes can be easily identified by the naked eye while most mutations are non-visible, which can only be detected biochemically or in an analogous way (Xin et al. 2008; Sestili et al. 2009; Chawade et al. 2010). So, if a favorable mutant trait was to be used in breeding practice, a marker for the favorable trait would be great help for its introgression since it reduces the number of necessary crosses and also ensures to eliminate as many random mutations as possible from the mutant lines. Such a marker could be visible, biochemical, or molecular (Sikora et al. 2011). As typical TILLING populations are based on the introduction of point mutations, it is relatively straightforward to track mutations through a subsequent crossing program using SNP markers (Dierking and Bilyeu 2009). This "molecular marker-assisted selection" (MMAS) can help the selection of target traits very early in the plant growth cycle and even from seeds, saving labor, time, and field space. In addition, the SNP-based MMAS in TILLING can also be achieved through automated and high-throughput screening in crop breeding

compared with conventional phenotypic selection. For instance, allele-specific molecular marker assays were developed for the soybean mutation identified by TILLING to reliably detect the inheritance of the mutant alleles and could be used in efficient breeding for desired seed phenotypes (Dierking and Bilyeu 2009).

However, some mutations, even if predicted to be deleterious do not necessarily affect overall gene function. Homologs of the target gene may still express, leading to functional complementation of the mutation. This is especially true for polyploid plants where homoeologs of the gene of interest may exist in all the genomes and when one allele is mutated, others may compensate for the loss. In practice, it is therefore often necessary to identify mutations in each homoeologous copy of the target gene and bring these together by crossing. Dong et al. (2009a, 2009b) screened the waxy genes *Wx-A1* and *Wx-D1* in 2,348 EMS-treated M_2 plants and found 121 mutants in total, then a complete waxy wheat was successfully bred by crossing two truncation mutants (*Wx-A1*-truncation and *Wx-D1*-truncation; the lines used were already null in *Wx-B1*) (Dong et al. 2009a, 2009b).

Additionally, highly mutagenized lines of polyploid species may require a number of backcrosses to purge potentially undesirable background mutations before they can be assessed for phenotypes or agronomic use, as also in diploid species where a much lower mutation rate will still generate phenotypes. The high mutation density in wheat TILLING population we reported previously (one mutation per 47 kb within the 16,000 Mb in hexaploid wheat genome) implies that any given individual is predicted to carry approximately 340,000 mutations (Chen et al. 2012). Thus, significant backcrossing would be needed to minimize the number of extraneous background mutations by backcrossing the mutants to wild type over several generations (Parry et al. 2009; Uauy et al. 2009; Slade et al. 2012). Otherwise the performance of the lines generated directly from crosses with the original mutants could conceivably be reduced by the background variations. When backcrossing, as mentioned above, the process can be accelerated through MMAS using the SNP itself as a molecular marker (Dierking and Bilyeu 2009).

Generally, a single TILLING population includes thousands of M_2 lines and it is a renewable reverse-genetic resource for continued analysis of many different genes of interest. However, this mutagenized population could be also used as a forward genetic tool to select individual mutants with improved traits for crop improvement. In TILLING, forward genetic mutant screening was only performed on M_2 (or M_3) families and M_3 seeds were stored until they were required for further analysis (Perry et al. 2003; Uauy et al. 2009; Knoll et al. 2011). However, in polyploid species, a large number of recessive mutations in single homoeologs of genes are less likely to show a phenotype. Whereas, there may be many exotic "visible" mutant phenotypes that were masked in M_2 (or M_3) plants, and their values could not be observed or less attended. To see the possible hidden phenotypes in our TILLING population (Chen et al. 2012), eight different M_3 mutants with similar phenotypes (dwarfing lines with late heading and shorter spike) were chose to backcross with the wild-type "Jinmai47." Then the visible mutant phenotypes of each BCF_2 segregating populations were recorded in reference to the wild type, the frequency of the distinguishable phenotypes was also estimated. As expected, a wide range of variations that are distinctive from the wild type and the mutant parents were found among the individuals of BCF_2 populations, and new noticeable phenotypes were also found in each population, such as wax leaf, more spikelet per spike and increased plant height, compared with the wild type and the eight mutant parents (Figures 4, 5, 6; Table 3). In the BCF_2 populations, individuals with higher plant height compared to the wild type and the mutant parents were found (with a 4% frequency averaged across the eight populations) though the eight original mutants were all dwarf ones. Moreover, there was no mutant with increased plant height compared with the wild type observed in the M_2 and M_3 populations. Through this backcross and selfing process, a new mutant phenotype (higher plant height) was observed in the BCF_2 population, which will update our TILLING phenotypic database and give more information to other researchers who are interested in plant architecture. Now, the various "novel" individuals provide us with new ideas and broader vision to use the TILLING resources, though it was known that TILLING allowed the identification of mutations that were silent in phenotyping. This small-scale forward strategy (to release the potential noticeable mutation phenotypes in several M_2 or M_3 plants) which is used conventionally in mutation breeding (MacKey 1954; Gaul 1958; He et al. 2001), providing the chance to see more target/interested traits before genotyping, which may in turn contribute to optimize the reverse-genetic strategy in the TILLING project (such as help to select candidate genes for TILLING based on the target/interested phenotype). It also showed the unique advantage offered by TILLING over the already available set of resources and techniques for functional genomics or crop breeding that the forward and reverse genetics can be conducted simultaneously to achieve mutual promotion in TILLING analysis. Figure 7 demonstrates the conventional procedure of TILLING in functional genomics and crop improvement. However, to date, there has been no TILLING-derived crop varieties that have been released, though there have been many successful examples of TILLING approaches in basic plant science. Good news was that some agricultural biotechnology company had been directing this technology into areas such as enhancing shelf life of tomato and reducing gluten content of wheat for sufferers of celiac disease (Parry et al. 2009). Importantly, the use of such novel alleles from TILLING in crops will not be hampered by the prohibitive legislation for GM crops; this alone should assure the rapid deployment of this approach in crop breeding.

However, forward phenotypic screening in TILLING for desired traits, such as grain quality, abiotic stress resistance, or photosynthesis-related physiological and biochemical traits, is not always easy. Traits with specific interest in practical breeding such as reduced height (dwarf), alterations in developmental phase (anthesis date), cuticle wax, awns, plant type, color (chlorophyll content), size, shape and attitude of leaf, spike morphology, size and color of grain, and also disease resistance and male sterility are easier to discover due to their visibility (Chawade et al. 2010; Chen et al. 2012; Dalmais et al. 2013). In this study, variants in plant height, spike morphology, leaf color, and heading date are the most visible phenotypes in the M_2 population (Chen et al. 2012). Moreover, in the BCF_2 populations, plant height, spike morphology, leaf morphology, and heading date are the most frequent types of

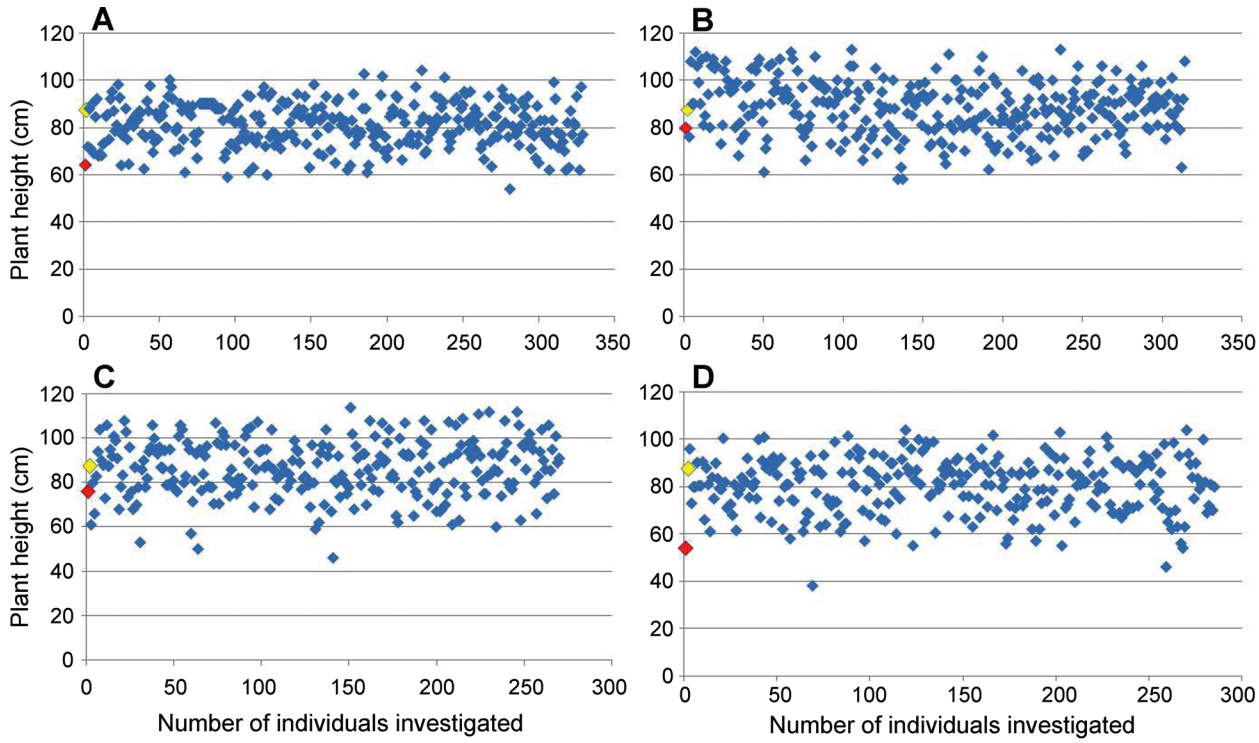


Figure 4. Plant height distribution in the four BCF_2 populations of Jinmai47 and its mutants
(A) 110-3 × Jinmai47. **(B)** 203-4 × Jinmai47. **(C)** 304-1 × Jinmai47. **(D)** 165-6 × Jinmai47. Plant height of the mutant parents was shown in red squares while the yellow squares show the height of the wild-type Jinmai47. Larger plant height compared to the wild type was identified in the BCF_2 populations though no individual was higher than the wild type in the M_2 or M_3 lines.

mutation (Table 3). This indicates that these visible various mutant traits in TILLING may be better alternatives for direct mutation breeding. Choosing the candidate genes related to these traits for TILLING could be a good strategy. However, TILLING is a reverse-genetic method allowing mutation screening of the target gene before any phenotyping. Genes involved in plant growth, grain quality, and abiotic stress resistance could be the target for TILLING analysis though it seems that new alleles related to quality traits are more likely to be achieved with TILLING than the quantitative traits due to its complex subsequent verification. Additionally, loss-of-function alleles is likely to be achieved more easily in TILLING population, suggesting that the breeding objectives target to remove unfavorable traits could be more suitable for TILLING (such as through knockout the GBSSI genes to produce a waxy wheat with little or no amylase) (Dong et al. 2009a), though TILLING can also provide gain-of-function mutants (Piron et al. 2010).

EcoTILLING: ASSESSING GENETIC VARIATION

EcoTILLING uses the same principle as TILLING to detect SNPs in genes in a natural population and has been confirmed to be efficient in discovering nucleotide polymorphisms in large populations (Comai et al. 2004; Wang et al. 2007; Galeano et al. 2009). All the technology that was used in the TILLING process for mutation screening can be adopted in EcoTILLING. However, for the traditional CELI-based EcoTILLING, the number of test materials in each pool needs to be reduced compared with that in TILLING where eight samples were usually pooled since the frequency of variations that existed in natural population was higher than those in artificially mutated populations, especially in polyploid species such as wheat with three sub-genomes. In practice, DNA pooling in EcoTILLING is usually conducted by mixing the genomic DNA of the test material and the control genotype in a 1:1 ratio (Comai et al. 2004; Chen et al. 2011).

Understanding and using genetic variation is very important for analyzing gene function, plant breeding, and conserving natural diversity. These tasks could be greatly facilitated by the availability of a large number of genomic sequences in public databases for many plant species. However, this large number of genome sequences was usually extracted from one or a few individuals or genotypes of each species, which provided only a starting point to understand how genomes vary in populations. Actually, sequencing even a subset of the genes in numerous individuals is expensive to date and it is difficult to obtain sequence information of multiple genotypes in the species. Fortunately, EcoTILLING provided a fast, cheap, and accurate method to recover a wide range of haplotype diversity in target genes in natural populations. In the EcoTILLING process, haplotype grouping

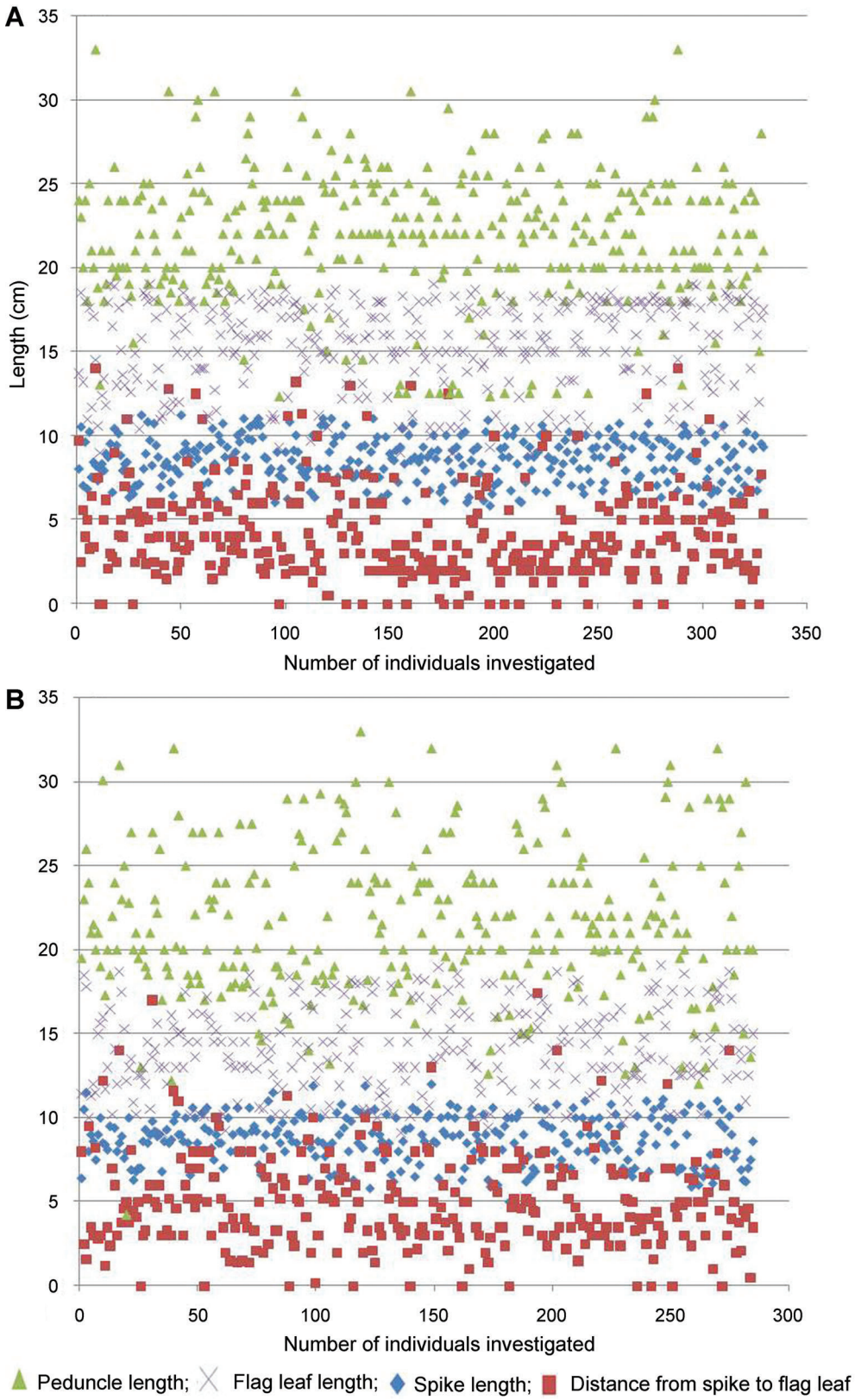


Figure 5. Distribution of the peduncle length, flag leaf length, spike length and distance from spike to flag leaf in the two BCF_2 populations

(A) 110-3 × Jinmai47. **(B)** 165-6 × Jinmai47. BCF_2 plants showed a wide range of variant phenotypes.

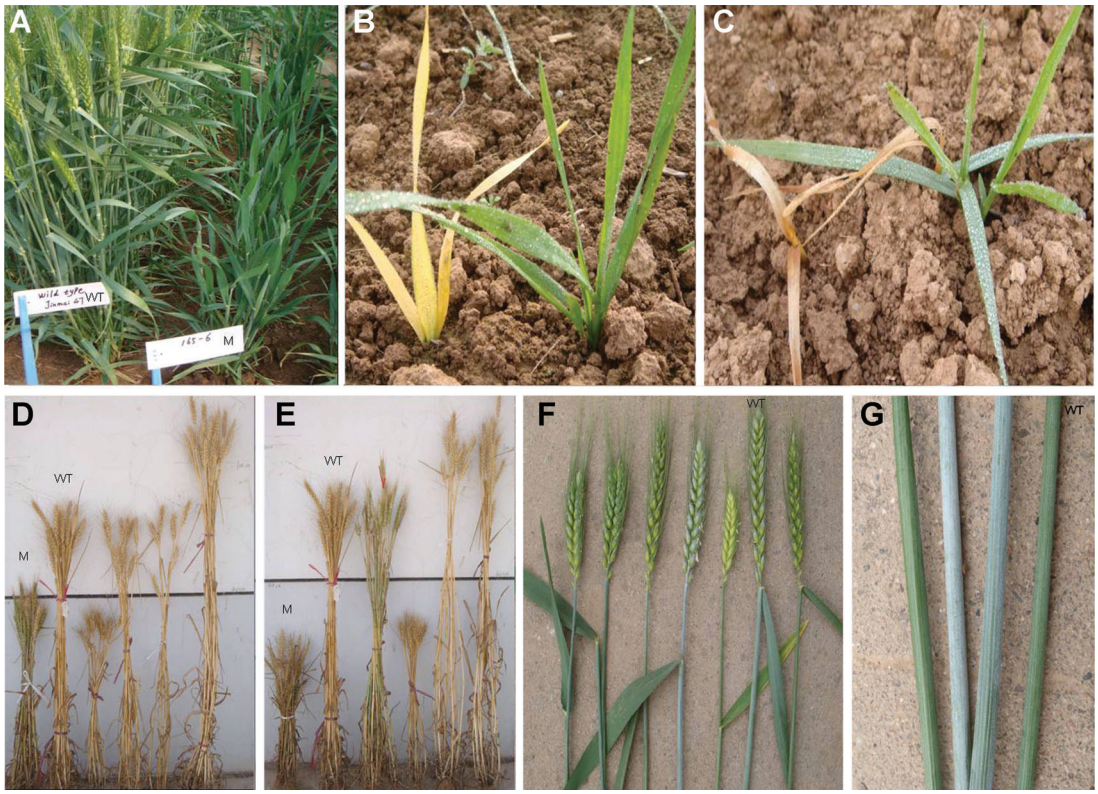


Figure 6. Mutant phenotypes observed in the BCF_2 plants
(A) The wild-type Jinmai47 and the mutant parent 165-6. **(B)** Albinism. **(C)** Early senescence. **(D and E)** Plant morphology in 110-3 × Jinmai47 and 165-6 × Jinmai47 populations. **(F)** Spike morphology and flag leaf pattern. **(G)** Wax leaf sheath. Many new exotic phenotypes were identified in BCF_2 populations that were not visible in the four mutant parents indicating that this small-scale backcross strategy was feasible for both forward genetic screens and for optimizing reverse-genetic strategies. M and WT show the mutant parents and the wild type.

was conducted firstly and then sequencing is only carried out on individuals or genotypes selected to represent each haplotype. EcoTILLING has been exploited for a wide range of applications including population diversity studies in *Arabidopsis*, rice, and potato (Comai et al. 2004; Kadaru et al. 2006; Elias et al. 2009); efficient analysis of allelic variations of Pina and Pinb genes to identify new alleles for future molecular breeding of wheat kernel hardness (Wang et al. 2008a) and on natural alleles of mlo and Mla in wild barley accessions for developing more durable resistance to powdery mildew disease (Mejlhede et al. 2006); association analysis to identify allelic variants controlling disease susceptibility in melon and drought tolerance in rice (Nieto et al. 2007; Yu et al. 2012); population structures and evolutionary relationships in *Brassica* species (Wang et al. 2010) and SNPs discovery and marker development in Common Bean (Galeano et al. 2009). Recently, in barley, 11 SNPs were detected and nine unique haplotypes were identified in *HSP17.8* (a heat shock protein) among 210 barley accessions by EcoTILLING approach. Four SNPs were significantly associated with related

Table 3. Frequency (%) of typical mutations observed among the four BCF_2 segregation populations investigated

Population*	Spike morphology	Lower fertility	Deep green leaf	Wax leaf	Flag leaf morphology	Albinism	Early senescence	Plant type	Late heading
1 (334)	70	1.0	20	68	25	0.5	–	92	35
2 (312)	85	2.0	5	45	16	–	0.1	97	65
3 (268)	30	–	18	20	32	0.3	–	88	55
4 (298)	75	0.5	34	17	22	–	0.5	95	46
M_2	0.61	0.19	0.08	0.04	0.15	0.27	0.08	1.92	0.27

*Here displayed four BCF_2 populations with the individual number of each population in the parentheses. M_2 shows the frequency of the mutations observed among the 2,610 M_2 individuals screened in this TILLING population (also see Chen et al. 2012).

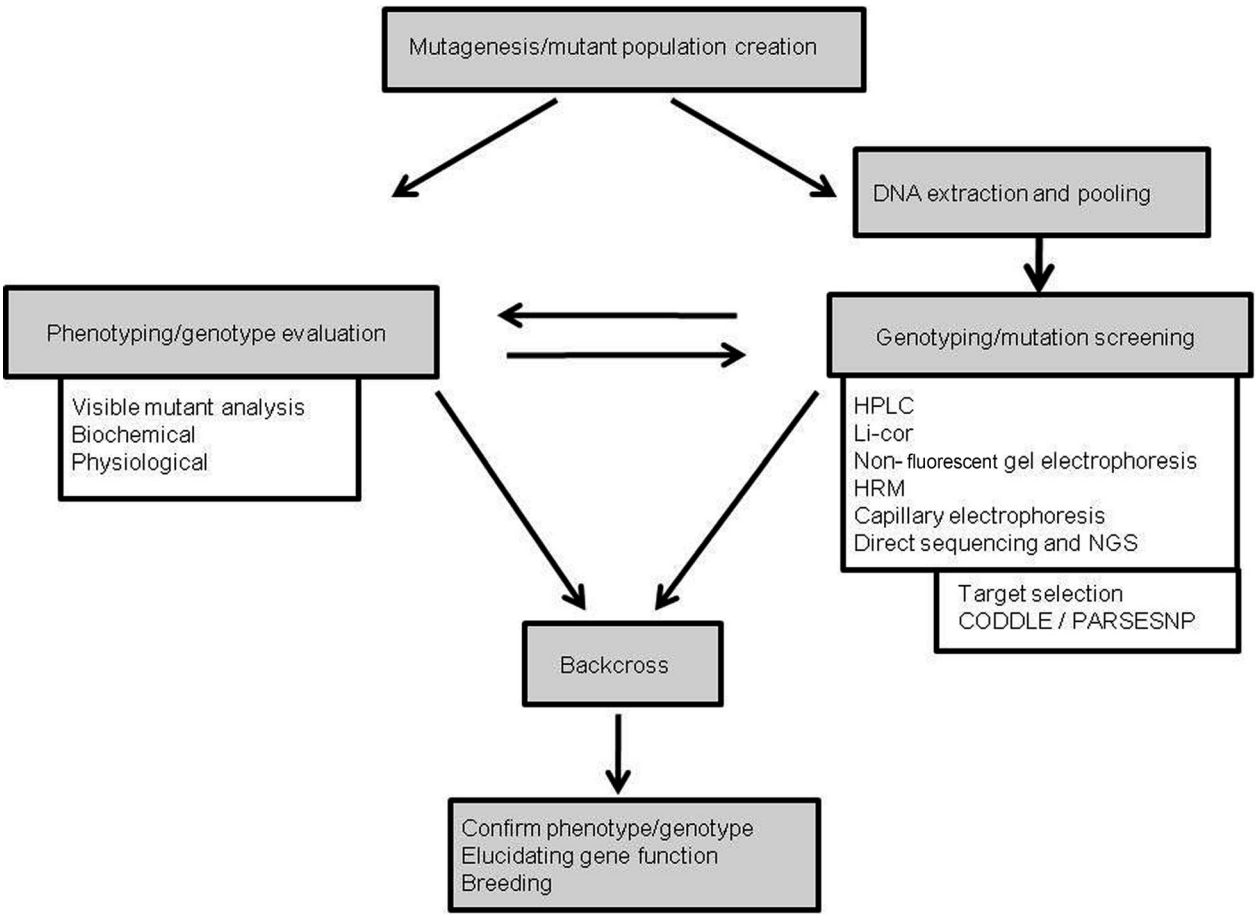


Figure 7. The flow chart of TILLING strategy
Reverse-genetic screens was performed on the M_2 DNA pools and forward genetic screens on the M_2 or M_3 families.

agronomic traits and can be used as DNA markers in marker-assisted selection to improve these agronomic traits, which provides new insights for barley abiotic tolerance study (Xia et al. 2013). In rice, Negrao et al. (2013) genotyped 392 rice accessions by EcoTILLING for revealing genotypic differences in five key salt-related genes, and 40 new allelic variants were identified in coding sequences. Association analyses showed that five SNPs significantly associated with salt-stress traits by affect gene function. Their results have uncovered allelic variants affecting salinity tolerance that is important in rice breeding (Negrao et al. 2013). Moreover, EcoTILLING in sugar beet reveals polymorphisms in the *BvFL1* gene that were associated with winter hardiness in beet (Frerichmann et al. 2013).

In general, the advantage possessed by EcoTILLING compared to the promising full sequencing approach is really the same as TILLING and this advantage will be greatly amplified if there were fewer haplotypes than individuals in the set (Comai et al. 2004; Wang et al. 2012). However, when the cost of re-sequencing with higher throughput are significantly decreased to a much lower level in the future, CELI-based EcoTILLING may be developed as sequeEcoTILLING (Weil 2009). Recently, association analysis emerged as a powerful approach to identify the role of genetic polymorphism in phenotypic variations, with the help of programgs, such as TASSEL v3.0 software (http://www.maizegenetics.net), and also the population structure analysis with STRUCTURE version 2 (http://pritch.bsd.uchicago.edu/structure.html), which allows EcoTILLING to be more feasible in crop improvement.

FUTURE PROSPECTS

During the past decade, large-scale genome sequencing projects have been completed in several major crop species (International Rice Genome Sequencing 2005; Schmutz et al. 2010; Brenchley et al. 2012; International Barley Genome Sequencing et al. 2012), and a huge volume of candidate gene sequence data are now available from public databases. Thus, the development of robust tools for identifying allelic series of mutations for gene functional studies and crop breeding has been desired by the research community. Therefore, the high-throughput TILLING method, or some strategy like it, will become increasingly popular, especially for agriculture, where non-transgenic methods are especially desirable. This mutant-based reverse-genetic approach may

shift the perception of genetic resources in agricultural studies, because it brings about not only loss-of-function mutants with various degrees of impairment but also provides gain-of-function mutants (Piron et al. 2010). These characters of TILLING for producing and identifying allelic series of mutations are very important for crop improvement. For wheat, expectantly, TILLING may achieve large progress in plant architecture and grain quality breeding in the first instance, through targeting the genes involved in gibberellin metabolic pathways or starch synthesis, as the information of these genes is relatively clear and some results have been achieved in this area (AL Phillips et al., unpubl. data, 2012) (Dong et al. 2009a; Slade et al. 2012). However, one negative factor is that, in contrast to technologies such as RNAi which, in principle, can target multiple genes simultaneously while point mutagenesis can only target a single copy of a group of related genes and mutations in different gene copies, may have to be brought together by crossing in order to achieve the desired effect. However, TILLING and similar techniques have, therefore, reawakened interest in random mutagenesis for crop improvement. Moreover, this promising strategy will be further enhanced by the explosion in genomic sequence data for crop species that benefit from the development of NGS and also the highly improved software and logistics. SequeTILLING/SequeEcoTILLING may instead of the traditional CELI-based TILLING/EcoTILLING strategies firstly in some large TILLING facilities in the near future. Recently, Uauy et al. (2013) showed their latest results on improving TILLING efficiency by combining genome capture with NGS, which aim to catalog gene sequence variation and provide an in-silico resource for researchers to study gene function and for breeders to deploy new variation in the field (Uauy et al. 2013). Overall, to improve food security, enhance productivities using "smart" crop varieties that yield more with fewer inputs is a viable option. For this, new technologies must be developed to promote fundamental research and practical breeding through improving genotyping and phenotyping methods. Thus, TILLING, which identifies novel alleles for functional genomics and crop improvement may become an effective tool for helping to address the challenge of feeding the twenty-first century world.

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