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REVIEW

TILLING, high-resolution melting (HRM), and next-generation sequencing (NGS) techniques in plant mutation breeding

Sima Taheri · Thohirah Lee Abdullah · Shri Mohan Jain · Mahbod Sahebi · Parisa Azizi

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Abstract Induced mutations have been used effectively for plant improvement. Physical and chemical mutagens induce a high frequency of genome variation. Recently, developed screening methods have allowed the detection of single nucleotide polymorphisms (SNPs) and the identification of traits that are difficult to identify at the molecular level by conventional breeding. With the assistance of reverse genetic techniques, sequence variation information can be linked to traits to investigate gene function. Targeting induced local lesions in genomes (TILLING) is a high-throughput technique to identify single nucleotide mutations in a specific region of a gene of interest with a powerful detection method resulted from chemical-induced mutagenesis. The main advantage of TILLING as a reverse genetics strategy is that it can be applied to any species, regardless of genome size and ploidy level. However, TILLING requires laborious and time-consuming steps, and a lack of complete genome sequence information for many crop species has slowed the development of suitable TILLING targets. Another method, high-resolution melting (HRM), which has assisted TILLING in mutation detection, is faster, simpler and less expensive with non-enzymatic screening system. Currently, the sequencing of crop genomes has completely changed our vision and interpretation of genome organization and evolution. Impressive progress in next-generation sequencing (NGS) technologies has paved the way for the detection and exploitation of genetic variation in a given DNA or RNA molecule. This review discusses the applications of TILLING in combination with HRM and NGS technologies for screening of induced mutations and discovering SNPs in mutation breeding programs.

Keywords Mutation breeding · Mutagenesis · EMS · TILLING · High resolution melting · Next-generation sequencing · SNPs

Abbreviations

BSA	Bulk segregant analysis
CEL I	Celery endonuclease
CE	Capillary electrophoresis

S. Taheri (✉) · T. L. Abdullah · P. Azizi
Department of Crop Science, Faculty of Agriculture, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia
e-mail: sima_taheri65@yahoo.com

T. L. Abdullah
e-mail: thohirah@upm.edu.my

P. Azizi
e-mail: bahar3236@yahoo.com

S. M. Jain
Department of Agricultural Sciences, University of Helsinki, PL-27, Latokartanonkaari 5, Helsinki, Finland
e-mail: mohan.jain@helsinki.fi

M. Sahebi
Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
e-mail: mahbod_sahebi@yahoo.com

M. Sahebi
Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

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CODDLE	Codons optimized to detect deleterious lesions
ComSeq	Compressed sequencing approach
dCARE	Deep candidate resequencing
DGGE	Denaturing gradient gel electrophoresis
dHPLC	Denaturing high performance liquid chromatography
EMS	Denaturing methanesulphonate
HDA	Heteroduplex analysis
HRM	High-resolution melting
IAEA	International Atomic Energy Agency; indels: insertion/deletion
MAB	Mutation-assisted breeding
MAF	Mutant allele frequency
MAS	Marker-assisted selection
mRNA	Messenger RNA
miRNA	microRNA
NGM	Next-generation mapping
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformational polymorphism
SSR	Simple sequence repeat
T-DNA	Transfer DNA
TGGE	Temperature gradient gel electrophoresis
TGS	Target genome sequencing
TILLING	Targeting induced local lesions in genomes
TPSeq	Targeted parallel sequencing
WGS	Whole genome sequencing

Introduction

One of the keys to sustainable agriculture is the creation of genetic variation in plant crops (Griggs et al. 2013). Over the past 70 years, mutation breeding has generated thousands of novel crop varieties in hundreds of crop species and billions of dollars in additional revenue, delivering higher yields, increased nutritional value and resilience to the effects of climate change, such as resistance to diseases and tolerance to drought. Mutation refers to sudden and heritable changes to the genetic material in any organism not caused by normal genetic segregation or recombination including spontaneous mutations and induced mutations (Suprasanna et al. 2015; Mba 2013; Austin et al. 2011; Oladosu et al. 2016). Spontaneous mutations occur in nature at a very low rate; therefore, physical and chemical mutagens are important for mutation induction in crop breeding programs. Mutations theoretically encompass all changes that occur in the DNA sequence and result in changes in the genetic code (van Harten 1998). An induced mutation is an alternative and complementary technique in plant breeding for the introduction of genetic changes and the establishment of new genetic resources. In the 1920s, Lewis Stadler at the University of Missouri discovered the mutagenic effects of X-rays in barley by exposing barley seeds. Since the 1950s, it has been widely used, specifically in crops with low genetic variability and those that are not amenable to improvement through conventional breeding methods. The number of physical and chemical mutagens used in mutation breeding is large and continues to increase (Mba et al. 2010; Mba 2013). According to the International Atomic Energy Agency (IAEA) Mutant Varieties Database (http://mvgs.iaea.org), 3233 mutant varieties of 214 different plant species have been officially released worldwide (Joint 2015). Of these, 49.5, 21.9, and 15% belongs to cereals, ornamental and decorative plants and legumes, respectively (Fig. 1). Gamma rays have been most used successfully to develop new mutant varieties of ornamentals with novel leaf and flower colour/shape (Taheri et al. 2014).

In any plant breeding program, genetic variation is necessary for crop improvement. Using mutagenesis to create novel genetic variation is extremely effective to promote natural genetic resources with the induction of random changes throughout the genome, resulting in a single plant with a large number of different mutations.

Chemical and physical mutagenesis has successfully assisted the development of improved and new cultivars among both seed and vegetatively propagated crops (Parry et al. 2009). Seeds of seed-propagated crops are treated with the mutagens while in vegetatively propagated plants stem cuttings, twigs, bulbs, buds, rhizomes and tubers are exposed to the mutagens. Chemical mutagens most often produce point mutations (Kodym and Afza 2003) and chromosome breaks that result in various chromosomal rearrangements that affect plant fertility and cause lethality. Among the different chemical mutagens, ethyl methane sulphonate (EMS) is the most powerful, effective, reliable and frequently used chemical mutagens in plants. Treatment with the mutagen breaks the nuclear DNA, and during the process of DNA repair, new mutations are induced randomly and are heritable. The changes can also occur in cytoplasmic organelles, again resulting in

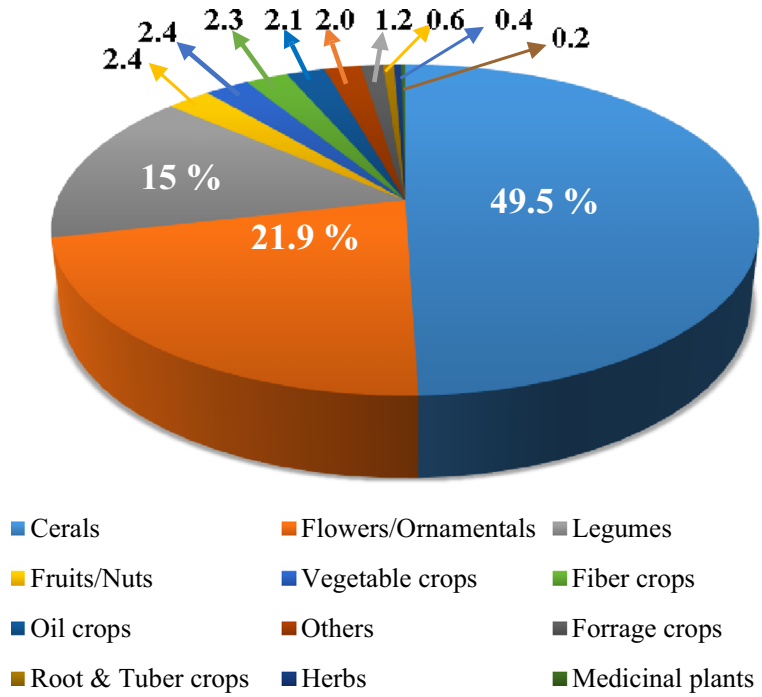

Fig. 1 Percentage (%) of mutant varieties by crop type reported on the Joint FAO/IAEA Mutant Varieties Database (Joint FAO/IAEA 2015)

chromosomal or genomic mutations that enable plant breeders to select useful mutants such as those with modified flower colour, flower shape, disease resistance and early flowering phenotypes (Jain 2010). Breeders are most interested in point mutations because large-scale changes can have negative effects on chromosome structures. However, large-scale changes in chromosome structure increase the number of recombination events, and the breaking of undesirable linkages is also highly valuable (Parry et al. 2009). EMS mainly produces C/G to T/A transitions with the induction of C to T substitutions, and EMS also generates G/C to C/G or G/C to T/A transversions or A/T to G/C transitions through 7-ethylguanine hydrolysis and through 3-ethyladenine pairing errors at a low frequency (Serrat et al. 2014). More recently, with biotechnology approaches such as in vitro cell and tissue culture and totipotency advantages, i.e. regeneration of the complete plant from individual plant cells, mutation breeding in vegetatively propagated plants has become more effective (Mba 2013). Various important factors affect the results of chemical mutagenesis, including the mutagen concentration, mutagen volume with respect to the sample size, treatment duration, temperature, pre-soaking of seeds, pH, use of catalytic agents and post-treatment handling of the mutants (Mba et al. 2010). Using induced mutations and enhancing mutation frequency rate and recombination of available desired genes from gene pools and the related plant species by sexual hybridization, plant breeders could successfully develop new cultivars with high yield and resistance to abiotic and biotic stresses. One of the advantages of mutation induction is the chance of obtaining unselected genetic variation when one or few characters of a prominent cultivar are to be modified to improve plants (Jain 2010). However, classical induced mutagenesis has some disadvantage, such as (1) the requirement of a large mutant population generation, (2) occurrence of chimeras, and (3) heterozygosity of the mutated loci. Mutation-assisted breeding (MAB) along with biotechnology approaches can improve these limitations without affecting the requirement of generating large mutant populations. Novel biotechnology approaches have made it feasible to survey specific regions of the genome that control a trait of interest for the introduction of alterations in large mutant populations with desirable genetic backgrounds (Mba 2013). This review briefly discusses the progress in the use of modern technologies such as targeting induced local lesions in genomes (TILLING) in combination with high-resolution melting (HRM) and next-generation sequencing (NGS) techniques for detection of mutations in mutation breeding programs to enhance the efficiency of induced plant mutagenesis in plant breeding era.

Forward and reverse genetic approaches for gene identification

Forward and reverse genetics are two main approaches that connect genotype to phenotype with the aim of determining the function of a gene/genes by screening the phenotype or genotype of individual mutants. In the classical genetics approach, known as forward genetics, mutant phenotypes (spontaneous or induced mutants) are compared with wild type to identify and characterize the causal genes.

In reverse genetics, the function of a gene identifies based on analysing the phenotype resulting from known gene sequence changes, in contrast to the reliance of forward genetics on identification of responsible genes for a specific phenotype (caused by induced mutagenesis or natural variation) (Jankowicz-Cieslak and Till 2015). A mapping population that segregates at the phenotype of interest is then produced, and candidate genes are mapped in a small region of the chromosome. Finally, the gene responsible for the desired phenotype will be identified by applying genetic engineering or reverse genetics methods (Esfeld et al. 2013; Ji 2013).

Despite the many identified responsible genes for the desired phenotype through the use of forward genetics, in most cases, genes of known sequence are not linked to a phenotype. This will happen more in non-model species that forward genetics can be more challenging due to genetic redundancy. In generated large mutant populations, single nucleotide polymorphism (SNP) discovery technologies are very useful for functional genomics, forensic medicine, clinical diagnostics, population genetics, molecular epidemiology and plant and animal breeding (Rigola et al. 2009). Advances in the genetic era, such as the discovery of DNA, the polymerase chain reaction (PCR) and genome sequencing, have led to techniques such as reverse genetic approaches (from genotype to phenotype), in which the phenotypic effect of the manipulation of a particular gene or gene product is assessed.

Classical reverse genetic approaches to identify induced mutations

Conventional and pre-screening mutation scanning methods in large mutant populations for the presence of sequence polymorphisms that require a separation step are discussed in this section. (1) The single-strand conformational polymorphism (SSCP) technique (Orita et al. 1989) performed under non-denaturing conditions results in the formation of single-stranded DNA sequence conformers with specific characteristic mobilities during polyacrylamide gel electrophoresis. Any changes in sequence results in conformer mobility change. (2) Denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla 1998) allows rapid screening for single-base changes in enzymatically amplified DNA. This technique is based on the migration of double-stranded DNA molecules through polyacrylamide gels containing linearly increasing concentrations of a denaturing agent. (3) Heteroduplex analysis (HDA) (Highsmith et al. 1999) is based on the detection of a mutation that results in conformational differences in the duplex DNA produced during polymerase chain reaction (PCR) amplification. The goal of heteroduplex analysis is to differentiate homoduplex DNA from heteroduplex DNA fragments based on their conformations under native conditions. (4) Denaturing high-performance liquid chromatography (dHPLC) (Underhill et al. 1997; Xiao and Oefner 2001) consists of the automatic detection of small insertions and deletions as well as single-base substitutions. In brief, produced heteroduplexes resulting from mixing, denaturing, and re-annealing two or more chromosomes that differ in sequence are retained less than their corresponding homoduplexes are in a unique DNA separation matrix. (5) In Temperature gradient gel electrophoresis (TGGE) (Li et al. 2002) a single chemical denaturant concentration is used to lower the melting temperature of all double-stranded molecules followed by the differential melting of the fragments with a temperature gradient. The sensitivity of a temperature gradient for DNA denaturation can be increased by establishing the gradient perpendicular to the direction of electrophoresis. These methods require a time-consuming step for the separation of PCR products in a gel or other matrix, which increases the risk of contamination in future reactions due to exposure of the PCR products to the environment. "SSCP, as a reverse genetic method, provides a high-throughput strategy for the detection of polymorphisms; however, it has low efficiency for the detection of novel mutations with a size limit of 200 to 300 bp in the target DNA sequence. Moreover, the high cost of performing a microarray and its low detection frequency of less than 50% (Borevitz et al. 2003; Tillib and Mirzabekov 2001) have limited its application.

TILLING: a reverse genetic technique for induced mutation detection

Sixteen years ago, the reverse genetic technique, targeting induced local lesions in genomes (TILLING) was first developed in Arabidopsis by McCallum and collaborators (McCallum et al. 2000a, b). TILLING is a high-throughput technique to identify single nucleotide mutations in a specific region of a gene of interest with a powerful detection method that resulted from chemical-induced mutagenesis. In the late 1990s, TILLING was first used by Claire McCallum, a graduate student (in cooperation with the Fred Hutchinson Cancer Research Centre and Howard Hughes Medical Institute), who worked on characterizing the function of two chromomethylase genes in Arabidopsis and applied reverse genetic approaches, such as T-DNA (incorporation of a foreign DNA into the genome of interest) lines and antisense RNA, but was unable to successfully apply these approaches to identify mutants for a DNA methyltransferase (chromotransferase) (Barkley and Wang 2008). The successful approach was what is now known as TILLING. Based on previous studies, TILLING can be applied in any species, regardless of its genome size and ploidy level and has been successfully applied in a range of crop plants such as hexaploid and durum wheat, barley, rice, tomato, maize, sorghum, soybean and potato, sunflower, melon, pea, and peanut to identify single base pair changes or small deletions in specific target genes (Uauy et al. 2009; Slade et al. 2005; Wang et al. 2012; Tsai et al. 2013; Singh et al. 2014; Sharp and Dong 2014) (see Table 1 for a list of those). Recently, Gauffier et al. (2016) used a TILLING approach in tomato to develop broad-spectrum resistance to potyviruses is prevented by eIF4E gene redundancy (Gauffier et al. 2016). For TILLING and for polymorphism analysis, the effect of missense mutations on the encoded protein must be evaluated. In this case, codons optimized to detect deleterious lesions (CODDLE) (http://www.proweb.org/coddle/) can be used to evaluate whether a missense mutation is likely to have an effect on the encoded protein in a selected specific DNA sequence with a length of 1 kb. CODDLE was developed as a general tool for polymorphism analysis, designing primers for any organism and detecting any mutagen based on the DNA sequence information input provided by users (De-Kai et al. 2006).

High-throughput TILLING comprised of a two-step procedure in which chemical mutagenesis (Koornneeff et al. 1982) is followed by a sensitive mutation detection instrument. For mutation induction, either biological agents such as transposons and T-DNA (Azpiroz-Leehan and Feldmann 1997; Balcells et al. 1991), or physical agents such as fast neutron, UV, x-ray and gamma-ray radiation (Stadler 1928; Sparrow and Woodwell 1962; Kovacs and Keresztes 2002) or chemical mutagens such as N-methyl-N-nitrosourea (MNU), 1,2:3,4-diepoxybutane (DEB) or ethyl methanesulfonate (EMS) can be used. Among these, T-DNA and transposon mutagenesis generate only destroyer mutants, whereas EMS mutagenesis is the most effective, reliable and powerful which produces different types of mutants within each gene, including nonsense, missense, splicing and cis-regulatory mutants (Krieg 1963; Kim et al. 2006; Kodym and Afza 2003; Greene et al. 2003; Brockman et al. 1984).

In TILLING, a variety of mutation scanning methods have been used for screening the mutants including CEL I endonuclease mutation detection together with electrophoresis conditions (Stemple 2004; Sato et al. 2006), high-resolution melting (HRM) (Botticella et al. 2011; Bovina et al. 2014; Sestili et al. 2015; Acevedo-Garcia et al. 2016) and next-generation sequencing (Tsai et al. 2011). The most favoured mutation detection method for TILLING in plants is the use of mismatch-specific celery nuclease, CEL I, together with the LI-COR gel analyser system (LI-COR Biosciences) (Gottwald et al. 2009). This technique was very popular in early 2000 to detect induced mutations caused by chemical mutagens. With the development of more sensitive and high-throughput techniques, it has been largely superseded.

With HRM, mutations are detected in target genes by using PCR, followed by denaturing and re-annealing of the double-stranded DNA (dsDNA) product which is monitored via a DNA-binding fluorescent dye. On re-annealing of the double-stranded DNA, fluorescence increases and results in high-resolution melt curve (temperature vs fluorescence) (Reed et al. 2007).

Recently, next-generation sequencing technique tremendously facilitated the discovery of mutations in TILLING populations that results in phenotypes, both for focused and for genome-wide discovery. The advent of next-generation sequencing as an important tool for whole-genome sequencing, re-sequencing and de novo sequencing has revolutionized genetic research (Quail et al. 2012). Next-generation sequencing in a single reaction can be used to sequence a large number of

Table 1 Applied mutation detection techniques and affected traits in model and crop plants

Species/ploidy level	Mutagen	Mutation detection technology	M_2 size	Mutation frequency (1 mutation/kb)	Traits	References
Arabidopsis (2×)	EMS	dHPLC, LI-COR	6912 3072 3712	1/170 1/300 1/89	– – –	Till et al. (2003a, b); Greene et al. (2003); Martín et al. (2009)
Maize (2×)	EMS	LI-COR	750	1/485	Chromomethylase	Till et al. (2004a, b)
Rice (2×)	EMS	LI-COR LI-COR CEL-I, Agarose gel	– 768 6912	1/1000 1/294 1/451	– – –	Wu et al. (2005); Till et al. (2007); Serrat et al. (2014)
Rice (2×)	EMS	TILLING	1860	–	–	Casella et al. (2013)
Rice (2×)	EMS	TILLING by Sequencing	2048	1/293	Phytic acid metabolism	Kim and Tai (2014)
Barley (2×)	EMS	dHPLC	9216	1/1000	Floral organ regulation	Caldwell et al. (2004)
Barley (2×)	EMS	LI-COR	10,279	1/500	Row type morphology and immunity to fungus	Gottwald et al. (2009)
Barley (2×)	NaN_3	TILLMore CEL-I. Agarose gel	5600	1/374	Starch metabolism	Talamè et al. (2008); Bovina et al. (2011); Sparla et al. (2014)
Wheat (6×)	EMS	TILLING-HRM	2020	1/26	Powdery mildew disease Resistance (TaMlo gene)	Acevedo-Garcia et al. (2016)
Wheat (6×)	EMS	TILLING by sequencing	4500	1/35000	–	King et al. (2015)
Wheat (6×)	EMS	LI-COR PAGE, LI-COR CEL-I, Agarose gel	10,000 1536 2348	1/24 1/38 1/37, 1/23	Starch quality Starch quality Starch quality and grain Hardness	Slade et al. (2005, 2012); Uauy et al. (2009); Dong et al. (2009a, b)
Wheat (6×)	EMS	LI-COR, HRM LI-COR	4500 4244	1/84 –	Starch quality Starch biosynthesis	Botticella et al. (2011); Sestili et al. (2010)
Wheat (6×)	EMS	Agarose gel, PAGE	2610	1/34; 1/47	Spike development	Chen et al. (2012)
Wheat (6×)	EMS	Direct sequencing	630	1/3	Grain hardness	Feiz et al. (2009)
Wheat (4×)	EMS	PAGE-LI-COR	8000 1386	1/40 1/51	Starch quality Starch quality	Slade et al. (2005, 2012); Uauy et al. (2009)
Wheat (4×)	EMS	TILLING-HRM	3992	–	Starch metabolism	Bovina et al. (2014); Sestili et al. (2015)
Wheat (4×)	EMS	CEL I, agarose gel, dHPLC	1140	1/77	Carotenoid metabolism	Colasuonno et al. (2016)
Wheat (2×)	EMS	CEL-I	1400	1/1300	Grain quality and lignin Biosynthesis	Rothe (2010)
Wheat (2×)	EMS	CEL-I	1532	1/92	Waxy and lignin	Rawat et al. (2012)
Sorghum (2×)	EMS	LI-COR	1600	1/526	Forage digestibility	Xin et al. (2008)
Sorghum (2×)	EMS	TILLING by sequencing (ComSeq)	1024	<0.1%	–	Nida et al. (2016)

Table 1 (continued)

Species/ploidy level	Mutagen	Mutation detection technology	M_2 size	Mutation frequency (1 mutation/kb)	Traits	References
Soybean (4×)	EMS	LI-COR	768; 529 40,000	1/550; 1/140 1/1000 to 1/1300	–	Cooper et al. (2008); Anai (2012)
Brassica rapa (2×)	EMS	LI-COR	1344	1/130.8, 1/41.5	–	Wang et al. (2008)
Brassica rapa (2×)	EMS	LI-COR	9216	1/60	DNA methylation	Stephenson et al. (2010)
Brassica oleracea (2×)	EMS	LI-COR	8750	1/447	Wax biosynthesis and dwarf stature	Himelblau et al. (2009)
Brassica napus (canola) (4×)	EMS	LI-COR - NGS	3158	1/109	Mutation identification in genes of interest	Gilchrist et al. (2013)
Lotus japonicus (2×)	EMS	LI-COR, CE	4904	1/502	Nodule development	Perry et al. (2003); Wang and Robson (2014)
Medicago truncatula	EMS	LI-COR	4500, 4350	1/485, 1/242.5	–	(Le Signor et al. 2009)
Sunflower (2×)	EMS	LI-COR	3651	1/475	Fatty acid biosynthetic pathway and downy mildew resistance	Sabetta et al. (2011)
Tomato (2×)	EMS	CE, HRM	8225	1/737	Proline biosynthesis	Gady et al. (2009)
Tomato (2×)	EMS	LI-COR	4741, 1926 4759 3052	1/322, 1/574 1/574 1/1237	Shelf life Virus resistance Shelf life	Minoia et al. (2010); Piron et al. (2010); Gauffier et al. (2016); Okabe et al. (2011, 2012)
Tomato (2×)	EMS	TILLING	3052	–	Ascorbate biosynthesis	Baldet et al. (2013)
Tomato (2×)	EMS	TILLING	–	–	Fruit set mechanisms	Mazzucato et al. (2015)
Tomato (2×)	EMS	LI-COR	–	–	Lycopene synthesis	Silletti et al. (2013)
Peanut (4×)	EMS	LI-COR	3420	1/967	Seed quality	Knoll et al. (2011)
Peanut (4×)	EMS	TILLING by sequencing	768	1/1066	Stress resistance	Guo et al. (2015)
Pea (2×)	EMS	LI-COR	8000	1/669	Gibberellin metabolism	Triques et al. (2007)
Flax	EMS	LI-COR	4894	–	–	Chantreau et al. (2013)
Cucurbita pepo	EMS	LI-COR	1464	–	–	Vicente-Dólera et al. (2014)
Brachypodium distachyon (2×)	NaN3	LI-COR	5530	1/396	Lignin biosynthesis	Dalmais et al. (2013)
Tobacco (2×)	EMS	TILLING by sequencing	3072	1/1423	Leaf yield	Reddy et al. (2012)

EMS ethyl methanesulfonate, *TILLING* targeting induced local lesions in genomes, *dHPLC* denaturing high performance liquid chromatography, *HRM* high-resolution melting, *NGS* next generation sequencing

DNA sequences. The high-quality DNA is sheared into fragments of a specific size and specific adapters are ligated to the 3′ and 5′ ends (Buermans and Den Dunnen 2014). These DNA templates are immobilized on a flow cell surface and amplified by sequential addition of nucleotides. Some of these techniques are applicable to many species, and each technique has advantages and limitations (Tables 1 and 2).

Table 2 Comparison of different methods involved in mutation detection in TILLING populations

Method	Advantages	Disadvantages	Reference
TILLING-CEL I	1. High sensitivity 2. High throughput 3. The populations generated for TILLING in any species provide valuable resources for teaching and research 4. Suitable for polyploids with higher mutation frequency	1. Rely on time and cost consuming enzymatic screening system 2. Needs multi-dimensional pooling 3. Only 5% of the total mutations in the EMS-mutagenized populations are truncations	Wang et al. (2012); Greene et al. (2003); Bleecker and Kende (2000); Byrne (2006); Eckardt (2007); Gilchrist et al. (2013); Parry et al. (2009); Perry et al. (2003)
TILLING-HRM	1. Non-enzymatic screening system 2. High sensitivity 3. Time and cost saving	1. Depends strongly on good PCR instruments and dyes. 2. Needs multi-dimensional pooling 3. Small insertions and deletions may be somewhat more difficult to detect than substitutions. (4) Detection sensitivity is limited to amplicons of <450 bp.	Reed and Wittwer (2004); Lochlainn et al. (2011); Wittwer (2009); Gady et al. (2009); van der Stoep et al. (2009); Simko 2016); Tindall et al. (2009); Chen et al. (2014a)
TILLING-NGS	1. Non-enzymatic screening system 2. High throughput 3. Time saving 4. Identification of mutations through targeted sequencing 5. More efficient in polyploids 6. Mutation detection in pools deeper than eight individuals	1. Cost is still high 2. Needs multi-dimensional pooling 3. High rate of incorrectly identified DNA bases in the sequence data produced, where billions of base calls translate to millions of errors which are not easily diagnosed or corrected. 4. The processing and storage of massive amounts of sequence data. Data analysis can be time-consuming and may require special knowledge of bioinformatics to garner accurate information from sequence data.	Zargar et al. (2015); Jünemann et al. (2013); Egan et al. (2012); Meacham et al. (2011); Ganal et al. (2009)

An overview of TILLING methodology

In the basic procedure of TILLING, seeds are mutagenized by treatment with ethyl methanesulphonate (EMS). The alkylating agent (EMS), which has great mutagenic potential, is the mutagen most commonly applied for almost all TILLING populations (Kurowska et al. 2011). Using the EMS mutagen, a high rate of mutation can be achieved that spreads out over the genome randomly without excessive DNA damage (Gilchrist and Haughn 2005). The developed M_1 plants are self-fertilized, and M_2 individuals are used to prepare DNA samples for mutational screening. The DNA samples are pooled and arrayed on microtiter plates followed by PCR amplification of the targeted DNA segment. Amplification products are incubated with a celery endonuclease (CEL I) that cleaves mismatches in heteroduplexes between mutants and wild type by preference. CEL I endonuclease is a member of the S1 nuclease family of single-strand-specific nucleases (Oleykowski et al. 1998) that cleaves on the 3′ side of mismatches and loop outs in heteroduplexes between wild-type and mutant DNA while leaving duplexes intact. Cleavage products generated by endonuclease are separated and electrophoresed using the LI-COR gel analyser system, and gel images are analysed with the aid of a standard commercial image-processing program (Adobe Photoshop; Adobe Systems, Mountain View, CA). Because mutations are detected on complementary strands, amplification products with different amounts of double end-labelling allow rapid visual confirmation and therefore can be easily distinguished from amplification artefacts. Upon mutation detection in a pool, the plant carrying the mutation is identified by screening individual DNA samples. The mutation is discovered by sequencing the target gene segment and identifying the type of nucleotide change (Lee et al. 2014). Figure 2 shows the general TILLING procedure.

Whereas the establishment of the initial screening population and the corresponding ordered DNA samples requires an investment of time and money, a

complementary approach to traditional TILLING, individualized TILLING (iTILLING), has been developed for Arabidopsis and is more cost and time effective (Bush and Krysan 2010). Furthermore, HRM as mutation detection method in TILLING populations (Botticella et al. 2011) with no reliance on enzymatic screening system or 'TILLING by sequencing' (Tsai et al. 2011) technologies have improved great potential of TILLING strategy for mutation detection which have been explained in the following sections.

Advantages and disadvantages of TILLING

The generated mutant population for any species using TILLING provides valuable resources for teaching and research. TILLING is an easy technique that does not require complicated manipulations or expensive equipment. It is used to easily screen point mutations to identify the functions of specific genes without the tedious tissue culture procedures that are involved in anti-sense RNA and RNAi. In addition, TILLING provides a high efficiency of mutation detection and high sensitivity attributed to the combination of CEL I, double-end fluorescent dye labelling and the LI-COR system as an alternative to dHPLC, as well as high frequencies of mutagenesis and a high-throughput screening capacity (De-Kai et al. 2006). Among reverse genetic techniques, TILLING can be used to identify genetic variability in either mutagenized or natural populations. In addition, unlike transgenic plants, for which there are some barriers to their creation or marketing, TILLING can be applied in plant research and breeding programs in various species because the variation does not involve the transformation of exogenous genetic material (Gilchrist et al. 2013). Using TILLING in a small and a large number of original research studies for a wide range of plants species (Table 1) supports the power of the TILLING technique for the investigation of genetic alterations in organisms with different genome sizes and ploidy levels. In contrast to the advantages of TILLING, it also has some limitations, such as the high cost and time commitment associated with the development of a mutagenized population for most species. Furthermore, the technique itself is labour-intensive, and the poor cleavage efficiency of the endonuclease CEL I and 59-39 exonuclease activity results in a reduction of signal/noise levels and prevents the performance of pooled sample analyses with more than eight samples per pool (Till et al. 2006). In addition, TILLING cannot provide information about the nature of the sequence changes and their possible effect on gene function; therefore, once mismatches have been detected, the results must be verified by sequencing, which incurs additional time and cost (Gilchrist and Haughn 2010) (Table 2). There are some bioinformatics tools such as the project aligned related sequences and evaluate SNPs (PARSESNP) program (http://www.proweb.org/parsesnp/) (Taylor and Greene 2003) to aid the researcher in deciding which mutations to characterize. Once TILLING process is completed, mutations are automatically analysed by the PARSESNP which provides graphical and tabular information on the location (PSSM difference) and severity (SIFT score) of mutations and provides information on the creation or loss of restriction sites caused by the induced polymorphisms. PSSM score ≥ 10 indicates a mutation that is more likely to have a damage effect on protein function. SIFT scores <0.05 have been empirically determined to be deleterious (Till et al. 2003a; Xin et al. 2008).

Some critical weak points of TILLING with CEL I endonuclease mutation detection method, as mentioned above, have resulted in the introduction of a diverse set of new screening platforms that do not rely on endonuclease mismatch cleavage, such as high-resolution DNA melting analysis (HRM), in which only simple PCR is performed before melting curve analysis, and next-generation sequencing which has recently been successfully applied in TILLING.

HRM: an alternative screening technique

HRM is a post-polymerase chain reaction (PCR) method that was established as an alternative screening technique for the detection of genetic variations (SNPs, mutations, methylation) in PCR amplicons (Zhou et al. 2005, 2004). Although dsDNA is very stable at room temperature, its two strands begin to separate with increasing temperature. The melting temperature (Tm) is the temperature at which 50% of the DNA is single-stranded. The length and guanine-cytosine (Ling et al. 2013) content of the DNA fragment affect Tm. The three hydrogen bonds of GC base pairs increase their stability compared with adenine–thymine (AT) base pairs linked by only two hydrogen bonds. Therefore, DNA sequences with a high GC content have a higher Tm than do DNA sequences containing a low number of GC base pairs (Druml and Cichna-Markl 2014). Melting of a short double-stranded region occurs at a faster rate if the regions melts in one transition without

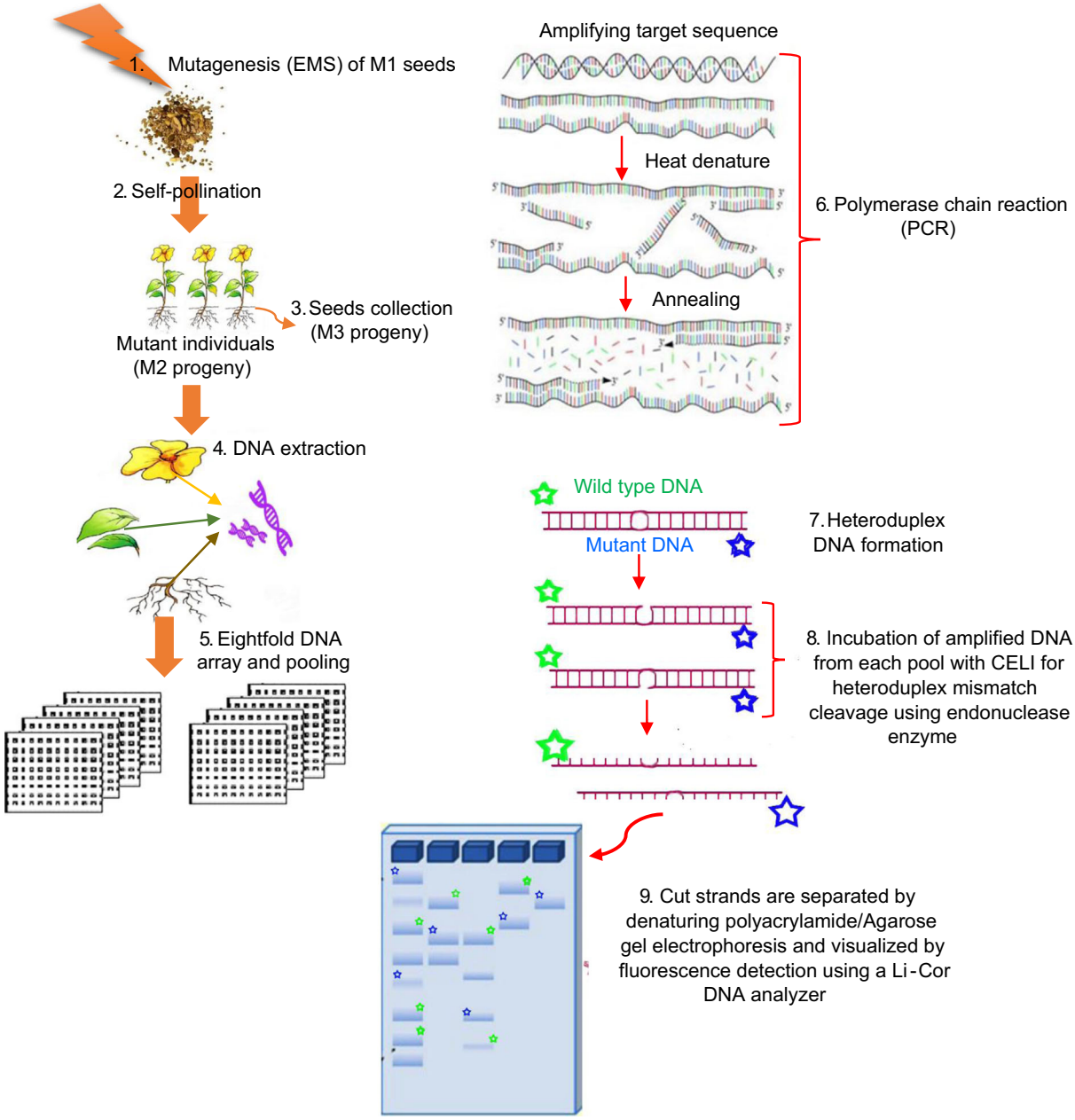

Fig. 2 Diagram of the TILLING strategy. TILLING procedure will be started by seed mutagenesis with a chemical mutagen and germinated to produce M_1 plants. M_1 plants are allowed to self-pollinate to generate M_2 plants. M_2 seeds will be collected for banking, and the DNA will be extracted from part of interest of plant for mutation discovery. Eightfold DNA pooling and DNA array in a two-dimensional format on 96-well plates. After PCR amplification of target genes with designed primers, upon heating and cooling steps, the heteroduplexes are formed and then incubated to digest with a mismatch endonuclease (CELI) for heteroduplex mismatch cleavage. Cut strands are separated by denaturing polyacrylamide/agarose gel electrophoresis and visualized by fluorescence detection using a Li-Cor DNA analyser. After identification of mutations, the polymorphic individuals are sent to sequencing to verify the induced mutation

intermediate states; in contrast, longer PCR products may melt in multiple stages or 'domains' (50–500 bps) (Erali and Wittwer 2010). HRM is a new screening method for DNA polymorphisms that was introduced in 2002 by a collaboration between academics (University of Utah, UT, USA) and industry (Idaho

Technology, UT, USA). Some benefits, such as simplicity, low cost, no need for enzymatic screening system and high sensitivity/specificity, make HRM an important new tool for genotyping, SNP polymorphisms, insertions or deletions (indels) and DNA methylations detection in PCR amplicons (Herrmann et al. 2007; White et al. 2007).

HRM working plan The HRM working plan is based on the changes in fluorescence along with the melting of double-stranded DNA that is measured using a saturated DNA-binding dye and a highly accurate optical detection system without the need for costly labelled probes. Three factors are involved in the HRM process: dye chemistry, instrument resolution and data analysis. HRM involves several main steps: (1) mutagenesis of plant material by EMS (seeds, pollen, leaf, flower, rhizome, root, among others), (2) DNA extraction, (3) PCR amplification with gene-specific primers labelled with a florescent dye, (4) high-resolution melting and (5) data analysis. First, PCR is performed in the presence of a dye that binds to double-stranded DNA (dsDNA). This dye emits low levels of fluorescence when unbound but is highly florescent in the bound state. Popular dyes for this application include CGreen/LCGreen Plus and SYBR Green I, which have a high sensitivity, stability, reliability and compatibility with many popular HRM instruments (Carén et al. 2006; Herrmann et al. 2006). However, the high cost of these dyes and the difficulties associated with the detection of heteroduplexes have limited their use (Pornprasert et al. 2008; Price et al. 2007; Worm et al. 2001). Recently, EvaGreen and ResoLight HRM dye (He et al. 2014) were found to be a less expensive saturated DNA binding dye with equal binding activity for GC-rich and AT-rich regions, without inhibiting amplification and no sequence preference (Wittwer 2009; Li et al. 2010). Following PCR, as the temperature is gradually increased, the changes in fluorescence in each sample during DNA melting are displayed by the temperature-normalized melting curves. The differences among curves demonstrate the relative difference in fluorescence (Δ Fluorescence, ΔF) of a respective sample in comparison to the non-mutant sample. A ΔF value of 0.05 is considered significant, according to the manufacturer's instruction (Li et al. 2010; Simko 2016; Hofinger et al. 2009). During HRM, even a subtle and small DNA variation that results in allelic differences among PCR amplicons can be detected based on melting curve differences. The demonstrated advantages of HRM analysis have resulted in the use of this approach to detect DNA polymorphisms in plant species such as apple, barley, grapevine, olive, almond, pepper, sweet cherry and *Solanum lycopersicum* L. (Liew et al. 2004; Wu et al. 2008; Chagné et al. 2008; Lehmensiek et al. 2008; Mackay et al. 2008; Donini et al. 2009; Gady et al. 2009; Ganopoulos et al. 2011; Golding et al. 2010). An exchange between G/C and T/A base pairs results in relatively large changes in Tm of approximately 0.8–1.4 °C (Liew et al. 2004). Duplex melting is generally monitored using intercalating dyes, although fluorescently labelled primers have also been used. HRM detection is based on the principle of melting curve changes during the process of DNA denaturation based on the primary sequence. The operation can be performed immediately after PCR without electrophoresis and in the same reaction tube. In comparison to electrophoresis, the detection time is reduced and the resolution is increased (Yu et al. 2013). Furthermore, HRM in combination with TILLING was used to screen an EMS-mutagenized wheat population to identify SNPs in hexaploid and durum wheat *SbeIIa* genes (Botticella et al. 2011; Bovina et al. 2014).

Advantages and disadvantages of HRM Among all the available scanning techniques, HRM is simple, rapid and inexpensive, and it is the only method that is performed in a closed tube and can be carried out in the same container used for PCR amplification in almost 15 min without need for any enzymatic screening system. The closed tube makes it possible to send the samples immediately after scanning for genotyping or sequencing (if necessary), without any need for processing or automation. In addition, contamination concerns are decreased when PCR products are not exposed to the environment (Reed and Wittwer 2004). However, the efficiency of HRM depends critically on the DNA concentration and quality, effective PCR, instrument decontamination and resolution and dyes (Herrmann et al. 2007). Some limitations, such as the GC content and length of the amplicon, affect the HRM efficiency, resulting in much shorter typical reads (only covering 150–500 bp) than those obtained with Li-Cor and CE. The usefulness of using HRM in the TILLING approach is highlighted when the target is a specific region with a known impact on protein structure or when the gene of interest contains many short exons and thus a short read length is acceptable. In addition, specialized software is

needed to analyse the different melt curves. In addition, sequencing of the amplicons is required to determine the number and position of mutations within the amplicon (Chateigner-Boutin and Small 2007). Other limitations of HRM are the difficulty associated with the detection of SNPs when the polymorphism occurs too close to either of the primer binding sites, the complex sequence, or the existence of a large number of alleles in the analysed population (Mader et al. 2008), the presence of multi-locus markers, the non-specific PCR amplicons (Distefano et al. 2012) and the presence of stutter bands (Dossett et al. 2010), which prevent reliable interpretation of the melting curve profiles. Nevertheless, HRM can be performed on standard qPCR machines with a simple software upgrade and thus is a suitable platform for initial TILLING screening (Sikora et al. 2012; Simko 2016) (Table 2). Future research should focus on improvements in hardware, software and DNA-binding dyes to increase the accuracy of the melting curves. Additionally, more research should be conducted to improve the HRM capacity to analyse longer amplicons, the reproducibility of the results and the possibility of results that can be transitioned across experiments and laboratories (Simko 2016).

Next-generation sequencing applications for causative EMS-generated mutation identification

Next-generation DNA sequencing technologies provide new opportunities for plant breeding programs. The detection and exploitation of genetic variation have always been an essential part of plant breeding. Next-generation sequencing techniques became commercially available in approximately 2005, providing great opportunities for the life sciences (Egan et al. 2012). Below, we will briefly describe a range of NGS applications in plant mutation breeding. Important applications are as follows.

Mapping by sequencing For many years, to identify genes and mutations in phenotype of interest, forward genetic screens (Page and Grossniklaus 2002; Candela and Hake 2008) has been applied as a powerful method for researchers. However, in classical forward genetic screens, mapping the casual mutation using genetic crosses is a complex and multistep procedure. Traditional mapping or positional cloning of mutations has been replaced by NGS using whole-genome sequencing (Smith et al. 2008; Srivatsan et al. 2008; Blumenstiel et al. 2009; Irvine et al. 2009; Lister et al. 2009; Schneeberger et al. 2009; Zuryn et al. 2010; Pawełkowicz et al. 2016). Ideally, the sequencing of mutant genomes is a simple way to identify mutations that cause phenotypes of interest. However, due to the numerous unrelated polymorphisms that segregate with the causative mutation in a mutagenized population, a very low signal is obtained. Therefore, some genetic analyses to purify the chromosomal location carrying the causative mutation are needed, even for NGS mapping approaches. Mapping by sequencing approach through NGS accelerated identification of casual mutations at SNP level even in complex genetic backgrounds (Schneeberger 2014; Schneeberger and Weigel 2011). After mutagenesis, mutation screening strategies depend on species, its breeding system, dominant or recessive nature of casual mutation. Nevertheless, mapping by sequencing is not dependent on reference genome sequences, genetic crosses and any kind of linkage information. In NGS mapping approaches, the background noise has been improved first by genetic analysis to refine the genomic region of interest or through bulk analysis of a very large number of mutant lines. In these years, several mapping by sequencing analysis pipelines have been introduced and have been applied in model plants (James et al. 2013), non-model plants (Nordström et al. 2013) and crop species like rice (Abe et al. 2012).

An approach called simultaneous mapping and mutation identification by deep sequencing (SHOREmap) by the principal of mutant allele frequency (MAF) estimation, including in isogenic backcross populations through whole-genome sequencing (WGS), was able to successfully map a causative mutation using Illumina Genome Analyser (GA) sequencing of a population of 500 pooled F_2 lines of Arabidopsis (Schneeberger et al. 2009). However, in that study, the strength of SHOREmap was not specified in fewer F_2 lines for mapping mutations with difficult-to-score phenotypes or working with organisms for which it is difficult to propagate large numbers. This straightforward method was applied in non-reference *A. thaliana* accession (Uchida et al. 2011, 2014) and in non-EMS mutations in non-model plants (Guo et al. 2012) to identify mutations that resulted in phenotypes of interest. The employed methodology was bulked segregant analysis (BSA), in which, after crossing a mutant to a different polymorphic accession, whole-genome sequencing data are extracted from pooled F_2 segregants and analysed for single nucleotide polymorphisms (SNPs). The

candidate SNPs are then detected and extracted using linkage analysis of the SNPs in the accessions used to produce the F_1 generation and filtering the linked SNPs with multiple appropriate criteria (Uchida et al. 2014). By contrast, in pools of mutant recombinants, dominant or semi-dominant mutations do not fix commonly.

Later, a DNA-based mapping-by-sequencing approach called next-generation EMS mutation mapping (NGM) by the principle of homozygosity mapping was developed by Austin et al. (2011) in *A. thaliana* using Illumina GA data to reliably and easily map candidate causative mutations even in small F_2 populations. Additionally, no prior mapping information is needed to perform this protocol (Austin et al. 2011). This tool is a Web-based service (http://bar.utoronto.ca/ngm) which (1) supports mutation mapping and identification and filtering for their effects and (2) visualizes chromosome-wide SNP densities and mapping scores, including manual refinement of mapping intervals. However, there is an uncertainty in the exact feature(s) of the NGM pipeline for mutant identification in fewer F2 populations.

Although described approaches are fast and facile, their application is affected by some obstacles including inter-accession crosses necessity that diminishes the success rate of genetic modifier screens and recognition of subtle phenotypic variation of mutants in F2 populations. Ashelford et al. (2011) applied re-sequencing of a novel Arabidopsis clock mutant early bird (ebi-1) genome that had been backcrossed four times to the parental line to create many candidate mutants. To reduce the cost of whole-genome sequencing of SHOREmap or NGM for large amounts of impractical and uninformative non-target regions, Liu et al. (2012) developed a time and cost-effective and facile targeted parallel sequencing (TPSeq) method without necessity of advanced computational devises and skills and identified three novel nitrate-signalling mutants in Arabidopsis, concurrently.

Furthermore, rice researchers developed another DNA-based mapping-by-sequencing method called MutMap by the principle of MAF estimation in backcross populations to use genome sequencing to identify the genomic position of EMS-induced mutants that are most probably harbouring agronomically important traits such as pale green leaves and semi-dwarfism (Abe et al. 2012). MutMap is based on the crossing of a M3-M5 mutant of interest directly to the original wild type and followed by selfing of F1 individuals, allowing clear segregation in second generation (F2) of subtle phenotypic differences. Then, DNA from 20 F2 individuals with mutant phenotype will send for Illumina whole-genome sequencing to detect casual SNPs different from the reference genome. Although this approach minimized the number of crosses in crop species and required mutant F2 progeny, it is not suitable for plants without reference genome sequence and mutants with early development lethality or sterility.

To reduce the number of causal candidate mutations, Hartwig et al. (2012), combined deep candidate resequencing (dCARE) using the new Ion Torrent Personal Genome Machine sequencing platform to identify causative mutations for the suppression of like heterochromatin protein1 (*lhp1*), a gene involved in chromatin-mediated gene repression. Notwithstanding that this method exploits backcross principle and SHORE pipeline for mapping analysis and necessity of targeted deep sequencing of candidates to isolate exact causative SNPs, it is a capable method for mapping-by-sequencing. Thereafter, Allen et al. (2013) screened leaf hyponasty EMS-induced mutants via single backcrossing of the mutant to its parent followed by whole-genome deep sequencing of F2 population and analysis with NGM approach to clearly identify causal mutation in HASTY gene involved in microRNA biogenesis. Avoiding MutMap obstacle, MutMap+ was developed for the identification of causal SNPs in rice mutants and gene isolation in crops that are recalcitrant to artificial crosses (Fekih et al. 2013).

Further studies on using next-generation sequencing for mutant identification have been done by Mateo-Bonmatí et al. (2014). They combined conventional linkage mapping and Illumina whole-genome re-sequencing to identify the causal mutations in four loss-of-function angulata (*anu*) mutants which affect deficits in leaf shape and pigmentation (Mateo-Bonmatí et al. 2014). Similarly, in Arabidopsis, next-generation sequencing of backcrossed bulk segregants was applied to identify abscisic acid (ABA)-resistant root elongation (AR) mutants (Thole and Strader 2015; Thole et al. 2014). Recently, Cheng et al. (2015) successfully applied next-generation sequencing in targeted mutation detection in hexaploid crop, *Crambe abyssinica*, to detect causative mutations in CaFAD2 gene leading to changes in crambe oil composition (Cheng et al. 2015).

SNP discovery through NGS SNP discovery is the most common application of NGS. SNPs have many applications in the construction of linkage mapping, genetic diversity analyses, association mapping and marker-assisted selection in several species (Cortés et al. 2011; Ray and Satya 2014; Thomson 2014; Huq et al. 2016). The discovery of SNPs using NGS has been reported in grapevine (*Vitis*) (Yang et al. 2016), cucumber (*Cucumis sativus* L.) (Pawełkowicz et al. 2016), pepper (*Capsicum*) (Devran et al. 2015), cabbage *(Brassica oleracea*) (Lee et al. 2015), olive tree (*Olea europaea* L.) (Kaya et al. 2013), sunflower (*Helianthus annuus* L.) (Pegadaraju et al. 2013), soybean (*Glycine max*) (Vidal et al. 2012), pepper (*Capsicum spp*.) (Ashrafi et al. 2012), wheat (Allen et al. 2011; Trebbi et al. 2011; Shavrukov et al. 2014), lupine (*Lupinus angustifolius* L.) (Yang et al. 2012), eggplant (Barchi et al. 2011), rice (Feltus et al. 2004; McNally et al. 2009; Yamamoto et al. 2010; Chen et al. 2014b; Zheng et al. 2016), Arabidopsis (Zhang and Borevitz 2009; Jander et al. 2002), barley (Close et al. 2009; Waugh et al. 2009), sorghum (Nelson et al. 2011), cotton (Byers et al. 2012), common beans (Cortés et al. 2011), soybean (Hyten et al. 2010), potato (Hamilton et al. 2011), flax (FU and Peterson 2012), *Aegilops tauschii* (You et al. 2011), alfalfa (Han et al. 2011), oat (Oliver et al. 2011), maize (Jones et al. 2009) and chickpea (*Cicer arietienum* L.) (Azam et al. 2012; Gaur et al. 2012) to name a few. Small plant genomes with a good reference genome, such as rice and Arabidopsis, are available in which SNP-derived NGS has been performed completely (Yamamoto et al. 2010; Ossowski et al. 2008). The quality of the SNP calling depends on factors such as the presence of repeat elements and incomplete or inaccurate reference genome sequences (Treangen and Salzberg 2012; Kumar et al. 2012).

TILLING by sequencing Although in TILLING a number of platforms has been applied for mutation detection of target genes within genomic DNA samples, most of these techniques rely on PCR amplification of target mutant region and wild type together to detect mismatches by heteroduplex amplicons using CEL I followed by gel electrophoresis or by HRM analysis. Such approaches are labour intensive and challenged by pools exceeding eight individuals, and screening a single target at a time is limited. Moreover, sequencing is required to characterize the mutations. 'TILLING by sequencing' technology demonstrates great potential by applying NGS in the TILLING strategy for mutation detection. An example of the use of Illumina sequencing in TILLING (Tsai et al. 2011; Rigola et al. 2009; Tsai et al. 2015; Granier et al. 2015) is the implementation of a flexible and effective combination of TILLING-NGS methods to detect mutations in targeted loci in rice and wheat. Likely, TILLING by sequencing technology was applied to identify mutations in genes to enhance leaf yield in tobacco (*Nicotiana tabacum*) (Reddy et al. 2012) and to detect mutations in genes related to biotic and abiotic stress resistance in peanut (*Arachis hypogaea*) (Guo et al. 2015). The method is characterized as follows: (1) TILLING-NGS is a high-throughput and reliable mutant identification method that is performed by the application of multidimensional pooling and a probability threshold, (2) it enables the identification of a mutation with an associated base change and effect, (3) it does not rely on labelled fluorescent primers or potentially variable endonuclease digestion, (4) it allows a flexible choice of pooling methods and species and (5) it is scalable in scope and experimental combinations.

In another study, Krothapalli et al. (2013) used next-generation sequencing in EMS-induced mutant of *Sorghum bicolor* defective in hydrogen cyanide release. They identified point mutation that resulted in a premature stop codon in the coding sequence of dhurrinase2, which encode a protein involved in the dhurrin catabolic pathway that is the cause of an acyanogenic sorghum mutant phenotype (Krothapalli et al. 2013).

Recently, Nida et al. (2016) applied a novel method to identify carriers of rare SNPs with frequency of less than 0.1% in TILLING population with compressed sequencing approach (ComSeq) in *S. bicolor*. ComSeq is a combination of NGS with new mathematical field of compressed sensing (CS) (Shental et al. 2010; Erlich et al. 2010). Compared to other TILLING population detection methods (Tsai et al. 2011; Missirian et al. 2011) which are based on 'multidimensional pooling' and detect only a single carrier, ComSeq detects many carriers without requiring sophisticated SNP calling methods (Missirian et al. 2011). Furthermore, this approach is a very cost-effective tool for breeders to identify novel alleles.

Mutation scanning by exon capture and next-generation sequencing Although TILLING by sequencing technique has improved traditional TILLING, because of the limit in the number of genes targeted in each run

and normalization of DNA samples at several stages, this method is still labour-intensive. In addition, despite declining costs of next-generation sequencing (Shendure and Ji 2008), it is still costly enough for researchers in eukaryotic species with large genomes. Fortunately, an alternative method was developed to focus on isolating of high-value coding gene sequences (exons) (comprising 2% of eukaryotic genome) and resequencing coding portions genome-wide of large genomes for SNP discovery (Hodges et al. 2007; Cosart et al. 2011). This exon capture method has been applied in durum wheat to identify variations in functionally important regions of the genome (Saintenac et al. 2011) and to identify SNPs within parents of mapping populations of bread wheat to allow the development of high-density maps (Winfield et al. 2012). Furthermore, Mascher et al. (2013) implied and evaluated a whole exome capture platform for cultivated barley and demonstrated its applicability to genome-wide variation discovery in related Hordeum species and hexaploid wheat (*Triticum aestivum*) (Mascher et al. 2013). Similarly, Henry et al. (2014) used exome capture and next-generation sequencing of rice TILLING mutants for large-scale mutation discovery. They also extended their study to EMS-induced mutations in tetraploid wheat showing the method is applicable for polyploidy species.

Lately, another study was performed for wheat TILLING evaluation using next-generation sequencing exon capture for mutation scanning (King et al. 2015). The above studies show that mutation detection by TILLING can be a less laborious, more efficient and less expensive method by using exon capture for genome re-sequencing in polyploid wheat, barley and other plant species. However, an improved genomic reference with more complete coverage of homologues is required for accurate mutation calling.

Like other technologies, NGS has some limitations, such as the requirement for costly equipment and reagents and the limited number of identified tags for sample barcoding, despite the large number of nucleotides that can be sequenced in one run. Analysis of the results of NGS sequencing is also challenging because it is necessary to distinguish between false-positive and real SNPs (Kurowska et al. 2011) (Table 2). In the near future, the number of NGS applications will certainly grow, and the available ones will improve. NGS technologies are paving the way toward a new era of scientific discovery and have great potential applications in plant-breeding programs for the development of superior cultivars of crops and ornamentals. As genome sequencing becomes easier, the additive demand of food will decrease in the coming decades for the next-generation plant breeders (Egan et al. 2012).

Conclusion and prospects

Mutagenesis has played a significant role in plant breeding. However, its effectiveness can be enhanced by using molecular technology and bioinformatics and assist plant breeders with useful induced mutations and develop mutation screening methods to identify novel alleles of target genes for crop improvement. Over the past 16 years, a variety of different techniques have been applied for detecting induced mutations in plant populations such as classical and recent reverse genetic techniques.

The TILLING-CEL I platform is widely applicable, but it involves steps such as endonuclease digestion reactions, cloning and gel electrophoresis runs, which are critical and also time-consuming. In addition, a lack of complete genome sequence information for many crop spices has slowed the development of suitable TILLING targets. In contrast, TILLING-HRM is accurate, sensitive, fast and cost-effective, and the only step required is a simple PCR performed prior to the DNA melting curve analysis. However, the number and position of mutations within the amplicon cannot be identified using this method alone. Therefore, amplicon sequencing is needed to identify sequence variants.

With the tremendous improvement of the cost and accuracy of NGS technologies in the past decade, the potential of TILLING-NGS approach for mutation detection has increased.

A comparison of mutation discovery by Illumina sequencing and traditional TILLING was conducted by Gilchrist et al. (2013). The sensitivity level of two approaches was found to be the same in oilseed rape, but sequencing was more expensive. They suggested several ways to decrease the cost of sequencing for mutation detection by barcoding samples during PCR amplifications (instead of post-amplification), using automated platforms for high complexity of PCR amplification and target many regions in multiple lines and applying platforms that provide more sequencing data per lane.

Improvements in target genome sequencing (TGS), TILLING by sequencing, mapping by sequencing and

targeted modification of specific genes will facilitate the discovery of SNPs and indels using NGS technologies to further assist our understanding of plant genetics and genomics.

In the future, NGS-enabled discoveries will continue in the next decade. Bioinformatics and sequence data will simplify the detection of target genes with allelic diversity resulting in the development of agriculture traits to meet the demands of global food insecurity. However, analysing large amounts of generated data after the application of NGS technologies, together with the extraction of bio-information, remains a challenge that needs to be addressed in future research.

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