Review

TILLING in Cereal Crops for Allele Expansion and Mutation Detection by Using Modern Sequencing Technologies

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Abstract: A substantial increase in yield of food crops is crucial to feeding the burgeoning global population. There is a need to introduce new breeding strategies that will accelerate the average phenotypic values of crop plants. The use of induced mutations coupled with modern genomics tools is an effective strategy for identifying and manipulating genes for crop improvement. High-throughput TILLING (Targeting Induced local Lesions IN Genomes) methodology, detects mutations in mutagenized populations, and EcoTILLING identifies single nucleotide polymorphisms (SNPs) within a natural population and associates these variations with traits of breeding interest. The main advantage of these techniques as a “reverse genetics” strategy is that they can be applied to any species regardless of genome size and ploidy level. In cereals, several space-induced and EMS-induced mutant populations have been used to identify mutants with important traits including salinity tolerance, grain size, and recombinant crossovers via TILLING by sequencing (TbyS). Genes such as *TaSSIV*, which plays an important role in starch granule formation, and *Pin a* and *Pin b*, which have been associated with kernel hardness in wheat, have been exploited in cereals via the EcoTILLING approach. This review focused on the functions and challenges of TILLING and the relation of TILLING to next-generation sequencing (NGS) technologies which help to exploit the induced mutations and their potential applications in cereal crops.

Keywords: TILLING; mutation breeding; cereal crops; TILLING by sequencing

1. Introduction

1.1. Background

As the world’s population continues to increase, food security is becoming an important issue in the 21st century. A recent evaluation suggested that to meet demands, cereal production must increase from current rates by 50% before the year 2030 [1,2], and the production should continue to increase by 70% until 2050. A related prediction suggested that to meet future demands for cereal production, the annual increase in crop production must rise by 37% [3]. It demonstrates that the possibility of the expected rise is questionable [2]. Moreover, this increase in productivity must be accomplished in the context of controlling unpredictable ecological and weather fluctuations [4].

There is a growing consensus that the key to achieving the required agricultural productivity will be to develop and exploit genetic variation in cereal crops [5]. However, within cereal crops, naturally occurring genetic variation is either low or primarily available in the form of distant relatives, and it is very difficult to harness this type of diversity for crop improvement.

Instead, modern plant breeding has been benefited from the use of consciously created germplasms in cereal crops which offer higher yield, nutritional value, and resilience to climate factors, including diseases [6–8]. These novel germplasms can result from the identification of useful mutations, which are changes in the genetic makeup induced using chemicals or radiation [7–10]. There are a number of physical (X-rays, gamma rays) and chemical (ethyl methane sulfonate, sodium azide) mutagens that are used on crop plants to create mutations which might increase crop productivity [3,9–13]. The International Atomic Energy Agency, one of the leading agencies working on the creation of mutant databases, reported that 3250 mutant varieties from a range of plant species have been formally released globally. Among these, 49.5% are cereals, with 21.9% and 15% being ornamental plants and legumes, respectively [10–14].

Recent research on cereal crops has yielded the genome sequences for many cereal varieties, providing a key foundation for identifying genetic variations associated with increased performance or improvement in average phenotypic values [15–17]. These genome sequence data for cereal crops can be used to identify new alleles in the germplasms which could help to improve important traits such as yield, disease resistance, and nutritional value [14,18]. The identification of beneficial mutations has been substantially advanced by the development of the TILLING (targeting induced local lesions in genomes) method [15,19]. TILLING enables rapid identification of mutations in genes of interest from within a mutagenized population, and has been applied to a wide range of crops including wheat, rice, maize, and sorghum in order to evaluate mutations in target genes [16,20].

While the general use of TILLING has been summarized in previous publications [21–23], the aim of the current review was to highlight recent advances and applications of TILLING and related methods (e.g., EcoTILLING) in cereals and polyploid species, including its use in combination with modern genomics approaches as TILLING by sequencing (TbyS).

1.2. TILLING and EcoTILLING

The TILLING method was developed in the late 20th century by Claire McCallum and colleagues while conducting experiments on *Arabidopsis* [24]. Initially, chromatography was used to separate the heteroduplex from wild-type and mutant homoduplex DNA strands, but this approach cannot be easily scaled up [19]. In order to increase throughput, a new process was developed that labeled individual PCR products in way that could be recognized by high-quality tools such as LI-COR (Lambda Instruments Corporation) DNA analyzers [25].

While TILLING has proven to be an effective method for identifying induced mutations, the EcoTILLING method can be used to evaluate naturally occurring variations. TILLING begins with the creation of a mutagenized population, which is then assessed for the presence of gene mutations linked to useful phenotypes. In contrast, EcoTILLING is used to identify polymorphisms from within a naturally occurring population of crop plants. Polymorphisms identified via EcoTILLING can be used to characterize phylogenetic diversity, and the technique can help to identify important alleles within cereal crops. EcoTILLING has been used to examine a range of crops, including *Arabidopsis*, rice, wheat, and sorghum, to identify new allelic variations for future molecular breeding [26,27]. For example, 192 diverse desi and kabuli chickpea accessions were evaluated using EcoTILLING to identify polymorphisms for 1133 transcription factors associated with seed weight [28]. Similarly, after identifying polymorphisms through EcoTILLING, association analyses were conducted to classify allelic variations underlying disease susceptibility in wheat and resistance to drought in rice [29]. An overview of previous research using EcoTILLING is provided in Table 1.

Table 1. Identification of natural polymorphism of target genes in different crops using EcoTILLING.

Species	Ploidy Level	Genotypes	Gene Name	No. of Genes	SNPs/Haplotypes	Methodology	Genome Size	Trait	Reference
<i>Arabidopsis thaliana</i>	2x	192	<i>DMMT2, DRM1C7, PIF2, AtWR</i>	5	55 (haplotypes)	CEL-1-PAGE	125 MB		[30]
<i>Triticum aestivum</i>	6x	214	<i>VRN-A1</i>	1		CJE/Agarose	17 GB	Vernalization	[31]
		1787	<i>Pin a, Pin b</i>	2	15	CEL-1-PAGE		Kernel hardness	[32]
<i>Oryza sativa</i>	2x	48	<i>MYB1, TPP, ADF</i>	14		CEL-1-PAGE/UL	400–430 MB		[33]
		48		3		Agarose gel			[34]
		45		87	-	CJE/Agarose gel		Boron toxicity	[35]
		375	<i>OSCP17</i>	1	38	CJE/Agarose gel		Salt tolerance	[36]
		95		19	14	CEL-1-PAGE		Drought tolerance	[37]
		392	<i>osCPK17, osRMC, osNHX1, osHKT1;5, Salt</i>	5	69	CEL-1-PAGE		Salt resistant	[38]
<i>Hordeum vulgare</i>	2x	512	<i>GBSSI, SSI, SSIIa, SSIIb, SBEIa, SBEIb</i>	6	23	EcoTbyS		Starch synthesis	[39]
		292	<i>Lhcb1</i>	1	23	CEL-1-PAGE	5.3 GB	Chlorophyll protein	[40]
		210	<i>HSP17.8</i>	1	11	CEL-1-PAGE		Heat shock protein	[41]
<i>Brassica sp.</i>	2x	117	<i>FAE1-A8, FAE1-C3</i>	2	18	CEL-1-PAGE	488–1544 MB 157 MB	Erucic acid content	[42]
	4x	187	<i>accD, matK, rbcL, atp6</i>	4	60	CEL-1-PAGE		Organelle genome	[43]
		676	<i>Chloroplast DNA</i>	1	538	EcoTbyS		Chloroplast DNA	[44]
<i>Solanum lycopersicum</i>	2x	49	<i>GCH1, ADCS, ADCL1, ADCL2, FPGSp, FPGSm, GGH1, GGH2, GGH3</i>	9		CEL-1-PAGE	950 MB	Folate biosynthesis	[45]
		127	<i>ACS2, CoPI, CYC-B, MSH2, NAC-NOR, PHoT1, PHYA, PHYB, PSY1</i>	9	54	CEL-1-PAGE		Plant development	[46]
<i>Gossypium hirsutum</i>	4x	277	<i>GhSus1At, GhSus1Dt, GhSus3At, GhSus4Dt, GhSus5Dt, GhSus6At, GhSus7Dt, GhSus8Dt</i>	8	24	CEL-1-PAGE	1724 MB	Sucrose synthesis	[47]
<i>Capsicum annum</i>	2x	233	<i>eIF4E, IIF(iso)4E, eIF(iso)4G, eIF4G</i>	4	62	CEL-1-PAGE	3.48 GB	Virus resistance	[48]
<i>Populus nigra</i>	2x	768	<i>CAD4, HCT1, C3H3, CCR7, 4CL3</i>	5	84	TbyS		Lignin biosynthesis	[49]
<i>Glycine max</i>	2x	25	<i>Gy1, Gy2, Gy3, Gy4, Gy5</i>	5	-	Agarose gel	1.15 GB	Seed protein	[29]
<i>Olea europea</i>	2x	96	<i>fad7</i>	1	3 (haplotypes)	CEL-1-PAGE		Fatty acid enzyme	[50]
<i>Beta vulgaris</i>	2x	268	<i>BTC1, BVFL1, BvFT1</i>	3	21	CEL-1-PAGE	714–758 MB	Winter hardiness	
<i>Jatropha curcas</i>	2x	907	<i>AF, EUO6, EFO3, DQ98, EU10, EU22, EU39, EU23, EU21, DQ15, DQ66, SUSY11</i>	12	86	CEL-1-PAGE	320.5 MB	Oil & stress tolerance	[51]
<i>Cicer arietinum</i>	2x	192		1133		Agarose gel	738 MB	Seed weight	[28]

TbyS = TILLING by sequencing, EcoTbyS = EcoTILLING by sequencing, CEL-1-PAGE = Celery enzyme – Polyacrylamide gel electrophoresis, MB = Megabyte, GB = Gigabyte.

1.3. Generation of Induced Mutation for TILLING

Several methods of chemically and physically inducing mutagenesis have been used to produce mutations in seed and vegetative crops. In the case of cereal crops, seeds are treated with the mutagen, and in the case of vegetative crops cuttings, bulbs, and tubers can all be treated with mutagens [52]. Different mutagens can create different kinds of mutations, enabling targeted creation of a variety of alleles. Breeders generally prefer to induce point mutations because higher scale changes are likely to adversely impact the structures of chromosomes, and ultimately the phenotypes [53,54]. However, higher scale alterations taking place in the structures of chromosomes can break some undesirable linkages, so these mutations could be valuable for crop breeding [52]. The most prominent and useful chemical mutagen is EMS (ethyl methane sulphonate). The results of TILLING depend on the effect of the chosen mutagen. The use of EMS produces a useful rate of mutation for the application of TILLING, saving time and resources [55].

The early TILLING method was first developed in the model plant *A. thaliana*, followed by *L. japonicus*. With improvements in technology, TILLING was quickly extended to a range of field and gardening crops. There are now various successful examples where TILLING has led directly to improvement in refinement, virus hostility, and maturing in melon, stiffer in potato, and natural products in sorghum [24,55].

1.4. Methods of Mutation Detection in TILLING

There are three key methods for screening mutant population using TILLING: first, the LI-COR method, which uses CEL I (celery enzyme) for mutation detection; second, high-resolution melting (HRM); and third, next-generation sequencing (NGS; Figure 1) [56,57]. Among all these methods, the preferred approach for TILLING in plants is LI-COR [58–60], a method developed in the early 2000s to investigate induced mutations that occur as a result of applying chemical mutagen [61,62]. High-resolution melting analysis is recent method for mutation detection [30,63]. The PCR reaction mixture gives higher resolution melt profiles using different temperatures that make it easy to detect SNPs in somatic mutations [64]. The proper balance of temperature and steady evaluation of fluorescence via instruments such as the Light Scanner System (Idaho Technology, Salt Lake City, UT, USA) or the Rotor Gene (Qiagen, Hilden Germany) enables individual mismatches to be detected in the target fragments from the mutant pools. HRM is a good choice when target genes have several small exons which are separated by large sequences of introns. HRM is also a quick assay, and is finished in a packed tube where there is no need for digestion or gel separation steps [30]. In hexaploid wheat, there are two main steps needed for the screening of mutation. The first involves a big fragment with many coding areas which is augmented using genome-specific primers, and the next step is HRM analysis, which uses specific primers for each exon [30].

Figure 1. Schematic workflow of TILLING by CEL-1 enzyme, TILLING by high-resolution melting, and TILLING by sequencing (TbS).

Recently, NGS (next-generation sequencing) methods have simplified the process of linking mutations identified in TILLING populations to relevant phenotypes [65]. The development of NGS has provided an essential tool for whole-genome sequencing and re-sequencing in the field of genetic research (Figure 1). In a recent study, lipoxygenase (*LOX*) genes were selected using TbyS and these genes showed higher expression in the seeds and roots of peanut. An M2 population was used to screen the 782 individuals, resulting in the identification of four missense mutations in *AhLOX*. Similarly, the phospholipase gene *AhPLD*, which plays an important role in drought and stress tolerance, was screened and three missense mutations were identified. Finally, *Arah1.01*, *Ara h1.02*, and *Ara h2.02*, which are involved in controlling the ratio of linoleic acid in seeds, were examined and six missense and three silent mutations were identified. These mutations were subsequently used to identify the functions of these genes [66]. TbyS is preferred compared to other methods for the following reasons:

1. The 3D pooling strategy allows for the observation of individual mutant plants and for the molecular recognition of mutation without requiring additional deconvolution of pools and additional sequencing steps;
2. TbyS can be used to recognize single base alterations and their impact on specific traits;
3. TbyS does not depend on fluorescent primers;
4. TbyS enables flexible options for pooling techniques.

Several important crops are polyploid, including bread and durum wheat, oilseed rape, and cotton [67]. Uauy et al. [68,69] found that despite this ostensible disadvantage, the numerous mutations of every gene made polyploid species suitable for TILLING, because the technique has the capability to bear very high mutation densities. The estimated mutation densities of diploid species are one mutation per 380 kb; however, this density increases to one mutation per 49 kb in tetraploids and one mutation per 32 kb in hexaploids (bread wheat and oat) [18]. The high mutation frequency seen in polyploid populations helps to recognize large allelic series in the target filtering of a comparatively smaller number of individuals [70]. If the mutant is utilized for breeding, these alterations can be efficiently minimized by backcrossing and choosing the mutation in each generation using SNP-based markers such as KASP [1,11,69].

2. Application of TILLING in Cereal Crops

Mutation breeding has been an important part of the agricultural process for eight decades. The development of the TILLING method has increased the variety of plants with improved yield not only via reverse genetic approaches, but also by helping to improve targeted breeding. The application of TILLING to mutation breeding using radiation or chemical treatment has resulted in higher yielding rice, barley, and wheat (Table 2) [70–72]. Some of the key applications of TILLING to modify important traits of cereal crops are described below.

2.1. TILLING for Starch Synthesis

The identification of the genes regulating the yield factor traits in different crops, followed by the establishment of novel cereal genomic resources and reverse genetics instruments, offers distinct possibilities for enhancing cereal yield [73–75]. In wheat, several relevant genes have been identified using mutant populations. For instance, in noodle production, fractional waxy wheat cultivars are preferred. The gene *SBEIIa* has been a focus of research, as suppression or expression of this gene affects amylose content. Using TILLING in wheat, 246 allelic sequences were identified in the waxy gene *SBEIIa* homoeologues. From these allelic sequences, 84 missense, 3 nonsense, and 5 split-site mutations were observed, with a mutation rate of one mutation per 40 kb. These alleles were combined using breeding, with the result that amylose in durum and bread wheat increased 47–55% compared to the WT [76].

Similarly, the *TaAGP* gene plays an important role in starch biosynthesis in the endosperm of wheat and also stimulates photosynthesis and carbon metabolism. There are four main missense

mutations recognized for *TaAGPL-B1*. Among these four, one mutation results in an amino acid change that affects grain starch content [77]. The *TaSSIV* gene is mainly involved in granule formation during starch synthesis. Fifty-four mutations were recognized in *TaSSIVb-D*, with a mutation density of one mutation per 165 kb. Three of the missense mutations and one nonsense mutation were found to have effects on protein function [16].

TbyS was also used to identify 37 *SBEI* mutations in a population of chemically induced mutant cultivar Nipponbare. *SBEI* plays a critical role in amylose content, and from these 37 identified mutations, one mutant M-4936 significantly reduced grain width and thickness. Physicochemical analysis revealed significant differences in apparent amylose content (15% vs. 19%) and protein content (9.2% vs. 4.8%) [78].

2.2. TILLING for Plant Architecture

In rice, TILLING was applied to an M2 population consisting of 961 mutant lines with the aim of examining nine target genes that play important roles in membrane transport proteins and the regulation of the salt-stress-tolerance mechanism. Forty-one mutants containing SNPs in the target genes were identified. The mutation frequency was one mutation per 1.5 kb, and the percentage of mutation per total sequence was 0.67%. Out of these 41 mutants, 9 had mutations in the exon region and of these, 7 were highly salt-tolerant [79]. In order to determine the stomatal limitation for photosynthesis, mutant rice was isolated and characterized for *SLAC1*. Using TILLING, four mutations of the *SLAC1* gene were identified in the population. Compared to WT, one mutation, named “*slac1*”, was associated with a low leaf temperature phenotype and high stomatal conductance with a high photosynthesis rate [80].

In a study of barley, two large mutant populations (*Hordeum vulgare* L.) were developed using EMS. Two main genes (*Hordoindoline-a* (*Hin-a*) and *Hordeum vulgare* Floral Organ Regulator-1 (*HvFor1*)) were evaluated and 10 SNPs were identified, 6 of which were missense mutations. After phenotyping the M3 generation, it was determined that 20% of the individuals had observable phenotypes [81]. The barley *HvD14* gene is involved in strigolactone signaling. TILLING was used to screen 6912 M2 individuals from the HorTILLUS population, and seven mutations of the *HvD14* gene were identified. One of these mutants, *hvd14.d*, was semi-dwarf and produced a significantly higher number of tillers compared to WT (Sebastian). This mutation altered the glycine at position 193 to glutamic acid. [82].

A mutagenized oat population created using EMS was developed to identify mutants through TILLING. Hundreds of mutations were identified in different genes, with a mutation rate of one mutation per 20–40 kb in the oat genome [83]. In a related study, the authors created 2600 mutagenized M2 individuals, which were reduced to 2550 in M3 seed lots. The M2 individuals were at first assessed by visual investigation for several phenotypes including a range of dwarfs to tall, early-flowering to late-flowering, and variations in leaf morphology and chlorosis. Phloroglucinol/HCl staining of M3 seeds from 1824 different M2 mutants identified multiple potential lignin mutants. These were further verified by evaluation using a quantitative approach [84].

Table 2. Overview of different TILLING approaches in various cereal crops.

Species	Ploidy	Mutagen	M ₂ Size	MF (Kb)	Mutation Detection Technology	Trait	Reference
Maize	2x	EMS	750	1/485	CEL-1- PAGE	Chromomethylase	[85]
Rice	2x	EMS	-	1/1000	CEL-1- PAGE		[32]
			768	1/294	CEL-1-PAGE		[86]
			6912	1/451	CEL-I -Agarose gel		[33]
		EMS	2048	1/293	TILLING by sequencing	Phytic acid metabolism	[87]
Barley	2x	EMS	9216	1/1000	dHPLC	Floral parts regulation	[81]
		EMS	10,279	1/500	CEL-1-PAGE	Fungus immunity	[88]
		NaN ₃	5600	1/374	CEL-I-Agarose gel	Starch metabolism	[89]
Wheat	6x 4x	EMS	2020	1/26	HRM	Resistance against powdery mildew	[62]
		EMS	4500	1/35,000	TILLING by sequencing		[90]
		EMS	10,000	1/24	CEL-1, PAGE	Quality of starch	[76]
		EMS	4500	1/84	CEL-1-PAGE, HRM	Quality of starch	[63]
		EMS	2610	1/34;1/47	Agarose gel, PAGE	Development of spike	[91]
		EMS	8000	1/40	CEL-1-PAGE	Starch quality	[92]
		EMS	3992		HRM	Starch metabolism	[59]
		EMS	1140	1/77	CEL I- Agarose gel, dHPLC	Carotenoid metabolism	[93]
		EMS	1532	1/92	CEL-I	Waxy and lignin	[94]
		EMS	733		Exome sequencing	Plant height	[6]
		EMS			TILLING by Sequencing, CEL-1- PAGE	Thousand grain weight	[95]
		EMS	10,000		TILLING by Electrophoresis	Gluten content	[96]
		EMS	1122		CEL-1-PAGE	Kernel hardness and starch	[97]

MF = Mutation frequency, EMS = Ethyl methane sulphonate, Kb = Kilobyte, HRM = High resolution melting, dHPLC = Denaturing high-performance liquid chromatography.

2.3. TILLING for Disease Resistance

Wheat production can be impaired by many biotic stress factors, with the powdery mildew caused by *Blumeria graminis* f. sp. *Tritici* being a major contributor. Therefore, there is a need to create resistant varieties through plant breeding. TILLING was used to identify partial loss-of-function alleles for the *TaMlo* gene, an orthologue of the barley *Mlo* gene. Natural and induced loss of function of barley *Mlo* caused durable resistance. In wheat, 16 mutations that resulted in amino acid changes were identified in three *TaMlo* homoeologues. The analysis clearly showed that mutants affected powdery mildew susceptibility. Four mutant lines that showed resistance were developed. These lines represent an important step towards the production of commercial non-transgenic, powdery-mildew-resistant bread wheat varieties [62]. Similarly, in maize, TILLING populations were screened for two genes (*ZmWAK-RLK1* and *ZmWAK-RLK2*) involved in resistance to northern corn leaf blight. There were a total of seven mutant lines for these two genes and they all resulted in amino acid substitutions. The mutants of *ZmWAK-RLK2* (*RLK2b*, *RLK2d*, and *RLK2e*) did not show any changes in susceptibility compared with WT. However, the mutants of *ZmWAK-RLK1* (*RLK1b*, *RLK1d*, and *RLK1f*) showed more susceptibility compared to WT. One *ZmWAK-RLK1* mutant, *RLK1e*, showed WT levels of disease resistance [98].

2.4. TILLING for Other Yield-Related Parameters

Barley is an essential crop with a hefty genome size of 5300 Mb. For use with TILLING, a mutant barley population was developed using a chemical mutagen (sodium azide). Identification of mutant alleles with TILLING revealed a significant role of the target genes in DNA repair and water logging [99,100].

In barley, two genes (*EDR1* and *NPR1*) were screened in a TILLING population. These genes regulate plant defense responses through the salicylic acid (SA) signaling pathway. Two missense mutation were identified from five total mutations [101]. In a related study, the “Sebastian” cultivar was subjected to mutagenesis to create a population that included 9600 lines in the M2 population. Thirty-two plant growth and development genes were targeted in a population screening. Through this screening, 382 mutations were identified, with a mutation density of one mutation per 477 kb. A total of 67 mutations were identified, of which 71% were missense and others were nonsense [71].

In a related study of oat, genomic DNA was extracted from M2 individuals and the mutation frequency was recorded. The average mutation frequency was one mutation per 20 kb as assessed by RAPD-PCR (random amplification of polymorphic DNA – polymerase chain reaction) fingerprinting, one mutation per 38 kb as assessed by MALDI-TOF (matrix assisted laser desorption/ionization – time of flight) examination, and one mutation per 22.4 kb as assessed by DNA aligning [97]. From the TILLING population, 520 lines were screened for differences in seed lignin levels. The lignin level of the screened population varied from 20 to 63 g per kg. The lignin level in the mutant lines increased significantly and the quality was also better than WT. The lines with low lignin levels in the seed showed high digestibility in rumen [33].

3. Prospect of TILLING

3.1. Next-Generation Sequencing and TILLING

TbyS was first developed by the Comai lab, and has improved the workflow of both TILLING and EcoTILLING. [61,68]. Since the early stages of the technique, a number of TbyS procedures have been modified to recognize point mutations from TILLING populations in an effective way that avoids the problems associated with traditional TILLING methods. Because DNA sequencing has become very affordable since the surge of NGS technologies, there has been an acceleration in the integration of these methods into the TILLING approach.

The cost of genotyping is another factor determining how and when NGS technology can be used with TILLING to improve mutation breeding. Crop breeding is critical for addressing the high

costs of a growing global population. At the same time, sequencing costs have rapidly decreased, contributing to a diffusion of NGS applications. Only 10 years ago, the sequencing cost for a million base pairs was approximately \$1000; the cost for the same sequence is now below \$0.10 [102]. The choice between complete- and partial-genome sequencing depends on the sensible utilization of funding [103]. The cost of WGS (whole-genome sequencing) for a single genotype of a three gigabase genome at 30× coverage is estimated at approximately \$5X. Targeted sequencing methods such as RAD-seq (restriction site associated DNA sequencing) have the capacity to model 200,000 SNPs in 100 individuals with a similar coverage at a 35-fold reduced cost compared to WGS for the same 100 individuals. If the complete genome sequence of a target species is available, then the cost can be lowered 10–14-fold by using processes like MSG (multiplexed shotgun genotyping) or GBS (genotyping by sequencing) [104]. Targeted sequencing is likely to be a better option for the exploration of large-scale markers, especially in the case of un-decoded genomes. The trend in sequencing follows Moore's law, which suggests that the cost of WGS or NGS will be rapidly reduced many-fold, and WGS may become the preferred option over partial-genome sequencing in near future [105]. The development of different high-throughput sequencing methods such as PacBio and Ion Torrent is helping to decrease the cost of sequencing, which will be valuable for TILLING platforms [102].

3.2. Importance of TbyS

TbyS was applied in tomato with the target gene *elF4E*, using Roche 454 technology in which the multidimensional pooling method was applied for 3000 M2 EMS mutagenized families and two mutations were identified. Moreover, for the same gene, 92 accessions of tomato were used to identify natural polymorphisms through TbyS and six haplotypes were discovered [106]. It was also determined that for cereal crops such as rice and wheat, using Illumina sequencing of target genes is helpful to find mutations in mutated population [107].

In the context of TbyS, there is a high-throughput sequencing technology in which specific regions of genes (exons) have been sequenced in order to determine the expressions of specific proteins that directly affect the phenotypes of the plants, a process known as exome-capture technology [108]. This methodology has been used in different cereal crops such as wheat and rice. This technology helps to sequence the coding region of mRNA for proteins and avoids sequencing non-coding regions of genes (introns), which mainly do not take part in protein function [69].

In order to examine TbyS results, multiple tools are used. MAPS (mutations and polymorphisms surveyor) is a popular tool for examining polyploid genomes because it uses every sample as a controlling agent to counter others and it enables differentiation between allelic variants as per the divergence among homoeologous and induced mutations [108]. There is a need to standardize the bioinformatic tools used to analyze the TbyS datasets to investigate desirable and accurate results.

TbyS technology has great potential for future applications. This technology was first used in tobacco to investigate the genes that play an important role in leaf yield [109], and in cereal crops to investigate genes linked with biotic and abiotic stress resistance [66]. Similarly, TbyS was used in sorghum with an induced mutant population [110]. In sorghum, there are enzymes which help to synthesize and catabolize cyanogenic glycosides (CG), which negatively impact humans and animals due to toxicity in the form the release of hydrogen cyanide (HCN). Dhurrinase1 and dhurrinase2 are enzymes responsible for releasing HCN in sorghum. EMS was used to induce mutations in 1000 M2 families, which were screened using TbyS. There was one point mutation which affected the premature stop codon sequence in the coding region of the dhurrinase2 enzyme, affecting the dhurrin catabolic pathway of the sorghum mutant. This mutant showed a different phenotype than WT. TbyS has also been used in polyploid species such as in camelina to improve the oil quality and non-nutritive traits [23]. Later, it was found that the three-dimensional method was used for polyploid and the investigation was done with an altered version of CAMBa, changed for the TILLING pooling scheme. This approach helped to improve the nutritional quality of crops by altering the seed oil characteristics and plant biomass [111,112].

3.3. Genome-Editing Technology and TILLING

Genome-editing technologies have grown rapidly, and play an important role in changing the sequence of nucleotides, such as specific changes in ORFs (open reading frames) or the introduction of a premature codon in the 5' region. There are different types of technologies used with crop plants, but the field is dominated by the use of CRISPR/Cas [113]. The major advantage of this technology is that it can create specific mutations in specific genes in diploid or polyploidy genomes. This can both generate functional alleles and replace mutant alleles [114]. In crop species, CRISPR/Cas has been used in rice and wheat to induce mutations [115] and there is a positive correlation with Cas9 expression level and mutation frequency [116]. In maize, this technology was used to induce targeted mutations in the protoplast with an efficiency of 13.1% [117]. CRISPR/Cas9 has been used to enhance resistance against blast by disrupting the ERF transcription factor in rice [118].

The main challenge to combining these genome-editing technologies and TILLING, which mainly edits or changes specific genes, is to enhance the effect on the phenotypic trait at desirable levels, and to limit the off-target variants which may or may not affect the phenotypic traits. Recently, both these technologies have been useful in analyzing point mutations for identifying single-locus heterosis [23]. Both technologies can be used to create allelic variations in the same gene; however, the outcome may be different because CRISPR/Cas uses only a few targets, while TILLING creates genome-wide mutations. For the improvement of wheat, it is necessary to understand the allelic diversity and homoeologous genes that control the phenotypes of different traits. Recent studies have shown that both technologies are important tools to understand these traits. [74,119,120]. The CRISPR/Cas system and TILLING were used to mutagenize each homoeologous gene copy in the cultivars Bobwhite and Paragon, respectively, for gene *TaGW2*. It was reported that *TaGW2* gene homoeologues showed negative regulators for grain size and thousand grain weight. Plants carrying single-copy nonsense mutations in different genomes showed different levels of grain size and weight, and thousand grain weight increased by an average of 5.5% (edited lines) and 5.3% (TILLING mutants). In any combination, the double homologue mutants showed higher phenotypic effects than the respective single-genome mutants. The double mutants had on average a 12.1% (edited lines) and 10.5% (TILLING mutants) higher thousand grain weight than WT lines. The highest increase in grain weight was shown for triple mutants of both cultivars, with increases of 16.3% (edited lines) and 20.7% (TILLING mutants) in thousand grain weight [95]. This example clearly demonstrates that both contemporary technologies have unique advantages and disadvantages, and their combined use may enhance the efficiency and accuracy of identifying targeted mutants for crop improvement.

4. Conclusions

TILLING coupled with NGS can be implemented to expand and detect allelic series of functional genes in cereals. High-throughput reduced-representation sequencing platforms like exome capture can detect these variations, which can be used in breeding and gene discovery. New gene-editing techniques like CRISPR/Cas have shown great promise in crop breeding, but while several high-throughput CRISPR/Cas protocols are optimized for use in cereals, they are limited to one or a few genes. TILLING creates uncontrolled genome-wide mutations. We argue that conscious integration of TILLING with CRISPR has the potential to make use of these two technologies to simultaneously create new alleles and efficiently edit functional genes that are of breeding interest. New and improved bioinformatics and analytical tools will be required to further reduce the time and improve the robustness of the procedure, especially when applying these technologies to polyploid cereals like wheat.

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References

- Allen, A.M.; Barker, G.L.A.; Berry, S.T.; Coghill, J.A.; Gwilliam, R.; Kirby, S.; Robinson, P.; Brechley, R.C.; D'Amore, R.; McKenzie, N. Transcript-specific, single-nucleotide polymorphism discovery and linkage analysis in hexaploid bread wheat (*Triticum aestivum* L.). *Plant. Biotechnol. J.* **2011**, *9*, 1086–1099. [CrossRef] [PubMed]
- Mba, C. Induced Mutations Unleash the Potentials of Plant Genetic Resources for Food and Agriculture. *Agronomy* **2013**, *3*, 200–231. [CrossRef]
- Tester, M.; Langridge, P. Breeding technologies to increase crop production in a changing world. *Science* **2010**, *327*, 818. [CrossRef]
- Godfray, H.C.; Garnett, T. Food security and sustainable intensification. *Philos. Trans. R. Soc. Lond.* **2014**, *369*. [CrossRef]
- Griggs, D.; Staffordsmith, M.; Gaffney, O.; Rockström, J.; Ohman, M.C.; Shyamsundar, P.; Steffen, W.; Glaser, G.; Kanie, N.; Noble, I. Policy: Sustainable development goals for people and planet. *Nature* **2013**, *495*, 305–307. [CrossRef] [PubMed]
- Mo, Y.; Howell, T.; Vasquez-Gross, H.; de Haro, L.A.; Dubcovsky, J.; Pearce, S. Mapping causal mutations by exome sequencing in a wheat TILLING population: A tall mutant case study. *Mol. Genet. Genomics* **2018**, *293*, 463–477. [CrossRef] [PubMed]
- Suprasanna, P.; Mirajkar, S.J.; Bhagwat, S.G. Induced Mutations and Crop Improvement. *Plant. Biol. Biotechnol.* **2015**, *593*–617. [CrossRef]
- Oladosu, Y.; Rafii, M.Y.; Abdullah, N.; Hussin, G.; Ramli, A.; Rahim, H.A.; Miah, G.; Usman, M. Principle and application of plant mutagenesis in crop improvement: A review. *Biotechnol. Biotechnol. Equip.* **2016**, *1*–16. [CrossRef]
- Mba, C.; Afza, R.; Bado, S.; Jain, S.M.; Davey, M.R.; Anthony, P. Induced mutagenesis in plants using physical and chemical agents. *Plant. Cell Cult. Essen. Methods* **2010**, *111*–130.
- Taheri, S.; Abdullah, T.L.; Jain, S.M.; Sahebi, M.; Azizi, P. TILLING, high-resolution melting (HRM), and next-generation sequencing (NGS) techniques in plant mutation breeding. *Mol. Breed* **2017**, *37*. [CrossRef]
- Li, H.; Rasheed, A.; Hickey, L.T.; He, Z. Fast-forwarding genetic gain. *Trends Plant. Sci.* **2018**, *23*, 184–186. [CrossRef] [PubMed]
- Kunter, B.; Bas, M.; Kantoglu, Y.; Burak, M. Mutation breeding of sweet cherry (*Prunus avium* L.) var. 0900 Ziraat. *Plant. Mutation Breed. Biotechnol.* **2012**, *453*–463.
- IAEA. Mutant Variety Database. Available online: <http://mvd.iaea.org/> (accessed on 2 March 2016).
- Jankowicz-Cieslak, J.; Mba, C.; Till, B.J. Mutagenesis for crop breeding and functional genomics. *Biotechnol. Plant. Mutation Breed* **2017**, *3*–18. [CrossRef]
- Newhouse, K.E.; Smith, W.A.; Starrett, M.A.; Schaefer, T.J.; Singh, B.K. Tolerance to imidazolinone herbicides in wheat. *Plant. Physiol.* **1992**, *100*, 882–886. [CrossRef] [PubMed]
- Guo, H.; Liu, Y.; Li, X.; Yan, Z.; Xie, Y.; Xiong, H.; Zhao, L.; Gu, J.; Zhao, S.; Liu, L. Novel mutant alleles of the starch synthesis gene *TaSSIVb-D* result in the reduction of starch granule number per chloroplast in wheat. *BMC Genomics* **2017**, *18*, 358. [CrossRef] [PubMed]
- Onda, Y.; Mochida, K. Exploring Genetic Diversity in Plants Using High-Throughput Sequencing Techniques. *Curr. Genomics* **2016**, *17*, 358. [CrossRef]
- Wang, T.L.; Uauy, C.; Robson, F.; Till, B. TILLING in extremis. *Plant. Biotechnol. J.* **2012**, *10*, 761–772. [CrossRef]
- Mccallum, C.M.; Comai, L.; Greene, E.A.; Henikoff, S. Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant. Physiol.* **2000**, *123*, 439. [CrossRef]
- Kumar, A.P.K.; Mckeown, P.C.; Boualem, A.; Ryder, P.; Brychkova, G.; Bendahmane, A.; Sarkar, A.; Chatterjee, M.; Spillane, C. TILLING by Sequencing (Tbys) for targeted genome mutagenesis in crops. *Mol. Breed.* **2017**, *37*, 14. [CrossRef]

21. Henikoff, S.; Comai, L. Single-nucleotide mutations for plant functional genomics. *Annu. Rev. Plant. Biol.* **2003**, *54*, 375. [[CrossRef](#)]
22. Kurowska, M.; Daszkowskagolec, A.; Gruszka, D.; Marzec, M.; Szurman, M.; Szarejko, I.; Maluszynski, M. TILLING—A shortcut in functional genomics. *J. Appl. Genet.* **2011**, *52*, 371. [[CrossRef](#)] [[PubMed](#)]
23. Kashtwari, M.; Wani, A.A.; Rather, R.N. TILLING: An alternative path for crop improvement. *J. Crop Improv.* **2019**, *33*, 83–109. [[CrossRef](#)]
24. Borevitz, J.O.; Liang, D.; Plouffe, D.; Chang, H.; Zhu, T.; Weigel, D.; Berry, C.C.; Winzeler, E.; Chory, J. Large-Scale Identification of Single-Feature Polymorphisms in Complex Genomes. *Genome Res.* **2003**, *13*, 513–523. [[CrossRef](#)] [[PubMed](#)]
25. Colbert, T.; Till, B.J.; Tompa, R.; Reynolds, S. High-throughput screening for induced point mutations. *Plant. Physiol.* **2001**, *126*, 480. [[CrossRef](#)] [[PubMed](#)]
26. Jawher, M.; Arabi, M.I.E.; MirAli, N.; Till, B.J. Efficient discovery of single-nucleotide variations in *Cochliobolus sativus* vegetative compatibility groups by EcoTILLING. *J. Plant Biochem. Physiol.* **2018**, *6*, 2.
27. Wang, J.; Sun, J.; Liu, D.; Yang, W.; Wang, D.; Tong, Y.; Zhang, A. Analysis of Pina and Pinb alleles in the micro-core collections of Chinese wheat germplasm by EcoTILLING and identification of a novel Pinb allele. *J. Cereal Sci.* **2008**, *48*, 836–842. [[CrossRef](#)]
28. Bajaj, D.; Srivastava, R.; Nath, M.; Tripathi, S.; Bharadwaj, C.; Upadhyaya, H.D.; Tyagi, A.K.; Parida, S.K. EcoTILLING-Based Association Mapping Efficiently Delineates Functionally Relevant Natural Allelic Variants of Candidate Genes Governing Agronomic Traits in Chickpea. *Front. Plant Sci.* **2016**, *7*, 450. [[CrossRef](#)]
29. Kadaru, S.B.; Yadav, A.S.; Fjellstrom, R.G.; Oard, J.H. Alternative EcoTILLING protocol for rapid, cost-effective single-nucleotide polymorphism discovery and genotyping in rice (*Oryza sativa* L.). *Plant. Mol. Biol. Reporter* **2006**, *24*, 3–22. [[CrossRef](#)]
30. Comai, L.; Young, K.; Till, B.J.; Reynolds, S.H.; Greene, E.A.; Codomo, C.A.; Enns, L.C.; Johnson, J.E.; Burtner, C.; Odden, A.R. Efficient discovery of DNA polymorphisms in natural populations by EcoTILLING. *Plant. J.* **2004**, *37*, 778–786. [[CrossRef](#)]
31. Liang, C.; Wang, S.Q.; Hu, Y.G. Detection of SNPs in the VRN-A1 gene of common wheat (*Triticum aestivum* L.) by a modified EcoTILLING method using agarose gel electrophoresis. *Aus. J. Crop. Sci.* **2011**, *5*, 318–326.
32. Ma, X.; Sajjad, M.; Wang, J.; Yang, W.; Sun, J.; Li, X.; Zhang, A.; Liu, D. Diversity, distribution of Puroindoline genes and their effect on kernel hardness in a diverse panel of Chinese wheat germplasm. *BMC Plant. Bio.* **2017**, *17*, 158. [[CrossRef](#)] [[PubMed](#)]
33. Rakshit, S.; Rakshit, A.; Matsumura, H.; Takahashi, Y.; Hasegawa, Y.; Ito, A.; Ishii, T.; Miyashita, N.T.; Terauchi, R. Large-scale DNA polymorphism study of *Oryza sativa* and *O. rufipogon* reveals the origin and divergence of Asian rice. *Theor. Appl. Genet.* **2007**, *114*, 731–743. [[CrossRef](#)] [[PubMed](#)]
34. Raghavan, C.; Naredo, M.E.B.; Wang, H.; Atienza, G.; Liu, B.; Qiu, F.; McNally, K.L.; Leung, H. Rapid method for detecting SNPs on agarose gels and its application in candidate gene mapping. *Mol. Breed.* **2006**, *19*, 87–101. [[CrossRef](#)]
35. Ochiai, K.; Shimizu, A.; Okumoto, Y.; Fujiwara, T.; Matoh, T. Suppression of a NAC-like transcription factor gene improves boron-toxicity tolerance in rice. *Plant. Physiol.* **2011**, *156*, 1457–1463. [[CrossRef](#)]
36. Negrão, S.; Almadanim, C.; Pires, I.; McNally, K.L.; Oliveira, M.M. Use of EcoTILLING to identify natural allelic variants of rice candidate genes involved in salinity tolerance. *Plant. Genet. Resour.* **2011**, *9*, 300–304. [[CrossRef](#)]
37. Yu, S.; Liao, F.; Wang, F.; Wen, W.; Li, J.; Mei, H.; Luo, L. Identification of rice transcription factors associated with drought tolerance using the EcoTILLING method. *PLoS ONE* **2012**, *7*, e30765. [[CrossRef](#)]
38. Negrão, S.; Almadanim, M.C.; Pires, I.S.; Abreu, I.A.; Maroco, J.; Courtois, B.; Gregorio, G.B.; McNally, K.L.; Oliveira, M.M. New allelic variants found in key rice salt-tolerance genes: An association study. *Plant. Biotechnol. J.* **2013**, *11*, 87–100. [[CrossRef](#)]
39. Raja, R.B.; Agasimani, S.; Jaiswal, S.; Thiruvengadam, V.; Sabariappan, R.; Chibbar, R.N.; Ram, S.G. EcoTILLING by sequencing reveals polymorphisms in genes encoding starch synthases that are associated with low glycemic response in rice. *BMC Plant. Biol.* **2017**, *17*, 13. [[CrossRef](#)]
40. Xia, Y.; Ning, Z.; Bai, G.; Li, R.; Yan, G.; Siddique, K.H.; Baum, M.; Guo, P. Allelic variations of a light harvesting chlorophyll a/b-binding protein gene (Lhcb1) associated with agronomic traits in barley. *PLoS ONE* **2012**, *7*, e37573. [[CrossRef](#)]

41. Xia, Y.; Li, R.; Ning, Z.; Bai, G.; Siddique, K.H.; Yan, G.; Baum, M.; Varshney, R.K.; Guo, P. Single nucleotide polymorphisms in *HSP17.8* and their association with agronomic traits in barley. *PLoS ONE* **2013**, *8*, e56816. [[CrossRef](#)]
42. Wang, N.; Shi, L.; Tian, F.; Ning, H.; Wu, X.; Long, Y.; Meng, J. Assessment of *FAE1* polymorphisms in three Brassica species using EcoTILLING and their association with differences in seed erucic acid contents. *BMC Plant. Biol.* **2010**, *10*, 137. [[CrossRef](#)] [[PubMed](#)]
43. Zeng, C.L.; Wang, G.Y.; Wang, J.B.; Yan, G.X.; Chen, B.Y.; Xu, K.; Li, J.; Gao, G.Z.; Wu, X.M.; Zhao, B.; et al. High-throughput discovery of chloroplast and mitochondrial DNA polymorphisms in *Brassicaceae* species by ORG-EcoTILLING. *PLoS ONE* **2012**, *7*, e47284. [[CrossRef](#)] [[PubMed](#)]
44. Qiao, J.; Cai, M.; Yan, G.; Wang, N.; Li, F.; Chen, B.; Gao, G.; Xu, K.; Li, J.; Wu, X. High-throughput multiplex cpDNA resequencing clarifies the genetic diversity and genetic relationships among *Brassica napus*, *Brassica rapa* and *Brassica oleracea*. *Plant. Biotech. J.* **2016**, *14*, 409–418. [[CrossRef](#)] [[PubMed](#)]
45. Upadhyaya, P.; Tyagi, K.; Sarma, S.; Tamboli, V.; Sreelakshmi, Y.; Sharma, R. Natural variation in folate levels among tomato (*Solanum lycopersicum*) accessions. *Food Chem.* **2017**, *217*, 610–619. [[CrossRef](#)] [[PubMed](#)]
46. Mohan, V.; Gupta, S.; Thomas, S.; Mickey, H.; Charakana, C.; Chauhan, V.S.; Sharma, K.; Kumar, R.; Tyagi, K.; Sarma, S.; et al. Tomato Fruits Show Wide Phenomic Diversity but Fruit Developmental Genes Show Low Genomic Diversity. *PLoS ONE* **2016**, *11*. [[CrossRef](#)] [[PubMed](#)]
47. Zeng, Y.D.; Sun, J.L.; Bu, S.H.; Deng, K.S.; Tao, T.; Zhang, Y.M.; Zhang, T.Z.; Du, X.M.; Zhou, B.L. EcoTILLING revealed SNPs in *GhSus* genes that are associated with fiber- and seed-related traits in upland cotton. *Sci. Rep.* **2016**, *6*, 29250. [[CrossRef](#)] [[PubMed](#)]
48. Ibiza, V.P.; Cañizares, J.; Nuez, F. EcoTILLING in *Capsicum* species: Searching for new virus resistances. *BMC Genomics* **2010**, *11*, 631. [[CrossRef](#)] [[PubMed](#)]
49. Marroni, F.; Pinosio, S.; Di Centa, E.; Jurman, I.; Boerjan, W.; Felice, N.; Cattonaro, F.; Morgante, M. Large-scale detection of rare variants via pooled multiplexed next-generation sequencing: Towards next-generation EcoTILLING. *Plant. J.* **2011**, *67*, 736–745. [[CrossRef](#)] [[PubMed](#)]
50. Sabetta, W.; Blanco, A.; Zelasco, S.; Lombardo, L.; Perri, E.; Mangini, G.; Montemurro, C. *Fad7* gene identification and fatty acids phenotypic variation in an olive collection by EcoTILLING and sequencing approaches. *Plant. Physiol. Biochem.* **2013**, *69*, 1–8. [[CrossRef](#)]
51. Maghuly, F.; Jankowicz-Cieslak, J.; Pabinger, S.; Till, B.J.; Laimer, M. Geographic origin is not supported by the genetic variability found in a large living collection of *Jatropha curcas* with accessions from three continents. *Biotechnol. J.* **2015**, *10*, 536–551. [[CrossRef](#)]
52. Parry, M.A.; Madgwick, P.J.; Bayon, C.; Tearall, K.; Hernandez-Lopez, A.; Baudo, M.; Rakszegi, M.; Hamada, W.; Al-Yassin, A.; Ouabbou, H. Mutation discovery for crop improvement. *J. Exp. Bot.* **2009**, *60*, 2817–2825. [[CrossRef](#)] [[PubMed](#)]
53. Cooper, J.L.; Till, B.J.; Laport, R.G.; Darlow, M.C.; Kleffner, J.M.; Jamai, A.; El-Mellouki, T.; Liu, S.; Ritchie, R.; Nielsen, N. TILLING to detect induced mutations in soybean. *BMC Plant. Biol.* **2008**, *8*, 9. [[CrossRef](#)] [[PubMed](#)]
54. Suzuki, T.; Eiguchi, M.; Kumamaru, T.; Satoh, H.; Matsusaka, H.; Moriguchi, K.; Nagato, Y.; Kurata, N. MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice. *Mol. Genet. Genom.* **2008**, *279*, 213–223. [[CrossRef](#)] [[PubMed](#)]
55. Serrat, X.; Esteban, R.; Guibourt, N.; Moysset, L.; Nogués, S.; Lalanne, E. EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. *Plant. Methods* **2014**, *10*, 5. [[CrossRef](#)] [[PubMed](#)]
56. Blomstedt, C.K.; Gleadow, R.M.; O'Donnell, N.; Naur, P.; Jensen, K.; Laursen, T.; Olsen, C.E.; Stuart, P.; Hamill, J.D.; Møller, B.L. A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production. *Plant. Biotechnol. J.* **2012**, *10*, 54–66. [[CrossRef](#)]
57. Piron, F.; Nicolai, M.; Minoia, S.; Piednoir, E.; Moretti, A.; Salgues, A.; Zamir, D.; Caranta, C.; Bendahmane, A. An induced mutation in tomato eIF4E leads to immunity to two potyviruses. *PLoS ONE* **2010**, *5*, e11313. [[CrossRef](#)]
58. Dahmani-Mardas, F.; Troadec, C.; Boualem, A.; Leveque, S.; Alsadon, A.A.; Aldoss, A.A.; Dogimont, C.; Bendahmane, A. Engineering melon plants with improved fruit shelf life using the TILLING approach. *PLoS ONE* **2010**, *5*, e15776. [[CrossRef](#)]

59. Sestili, F.; Botticella, E.; Bedo, Z.; Phillips, A.; Lafiandra, D. Production of novel allelic variation for genes involved in starch biosynthesis through mutagenesis. *Mol. Breed.* **2010**, *25*, 145. [[CrossRef](#)]
60. Henikoff, S.; Till, B.J.; Comai, L. TILLING. Traditional Mutagenesis Meets Functional Genomics. *Plant. Physiol.* **2004**, *135*, 630–636. [[CrossRef](#)]
61. Tsai, H.; Howell, T.; Nitcher, R.; Missirlian, V.; Watson, B.; Ngo, K.J.; Lieberman, M.; Fass, J.; Uauy, C.; Tran, R.K. Discovery of Rare Mutations in Populations: TILLING by Sequencing. *Plant. Physiol.* **2011**, *156*, 1257. [[CrossRef](#)]
62. Acevedo-Garcia, J.; Spencer, D.; Thieron, H.; Reinstädler, A.; Hammond-Kosack, K.; Phillips, A.L.; Panstruga, R. *mlo*-based powdery mildew resistance in hexaploid bread wheat generated by a non-transgenic TILLING approach. *Plant. Biotechnol. J.* **2017**, *15*, 367. [[CrossRef](#)] [[PubMed](#)]
63. Botticella, E.; Sestili, F.; Hernandez-Lopez, A.; Phillips, A.; Lafiandra, D. High Resolution Melting analysis for the detection of EMS induced mutations in wheat *Sbella* genes. *BMC Plant. Biol.* **2011**, *11*, 156. [[CrossRef](#)] [[PubMed](#)]
64. Li, Y.D.; Chu, Z.Z.; Liu, X.G.; Jing, H.C.; Liu, Y.G.; Hao, D.Y. A cost-effective high-resolution melting approach using the EvaGreen dye for DNA polymorphism detection and genotyping in plants. *J. Integrat. Plant. Biol.* **2010**, *52*, 1036–1042. [[CrossRef](#)] [[PubMed](#)]
65. Quail, M.A.; Smith, M.; Coupland, P.; Otto, T.D.; Harris, S.R.; Connor, T.R.; Bertoni, A.; Swerdlow, H.P.; Gu, Y. A tale of three next generation sequencing platforms: Comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* **2012**, *13*, 341. [[CrossRef](#)] [[PubMed](#)]
66. Guo, Y.; Abernathy, B.; Zeng, Y.; Ozias-Akins, P. TILLING by sequencing to identify induced mutations in stress resistance genes of peanut (*Arachis hypogaea*). *BMC Genomics* **2015**, *16*, 157. [[CrossRef](#)] [[PubMed](#)]
67. Adamski, N.M.; Borrill, P.; Brinton, J.; Harrington, S.; Marchal, C.; Uauy, C. A roadmap for gene functional characterisation in wheat. *PeerJ* **2018**, 2167–9843.
68. Irshad, A.; Guo, H.; Zhang, S.; Gu, J.; Zhao, L.; Xie, Y.; Xiong, H.; Zhao, S.; Ding, Y.; Ma, Y. EcoTILLING Reveals Natural Allelic Variations in Starch Synthesis Key Gene *TaSSIV* and Its Haplotypes Associated with Higher Thousand Grain Weight. *Genes* **2019**, *10*, 307. [[CrossRef](#)]
69. Uauy, C.; Wulff, B.B.; Dubcovsky, J. Combining Traditional Mutagenesis with New High-Throughput Sequencing and Genome Editing to Reveal Hidden Variation in Polyploid Wheat. *Ann. Rev. Genetics* **2017**, *51*, 435–454. [[CrossRef](#)]
70. Chen, L.; Hao, L.; Parry, M.A.; Phillips, A.L.; Hu, Y.G. Progress in TILLING as a tool for functional genomics and improvement of crops. *J. Integr. Plant. Biol.* **2014**, *56*, 425–443. [[CrossRef](#)]
71. Szurman-Zubrzycka, M.E.; Zbieszczak, J.; Marzec, M.; Jelonek, J.; Chmielewska, B.; Kurowska, M.M.; Krok, M.; Daszkowska-Golec, A.; Guzy-Wrobelska, J.; Gruszka, D. HorTILLUS—A Rich and Renewable Source of Induced Mutations for Forward/Reverse Genetics and Pre-breeding Programs in Barley (*Hordeum vulgare* L.). *Front. Plant. Sci.* **2018**, *9*, 216. [[CrossRef](#)]
72. Gilchrist, E.; Haughn, G. Reverse genetics techniques: Engineering loss and gain of gene function in plants. *Brief. Funct. Genomics* **2010**, *9*, 103. [[CrossRef](#)] [[PubMed](#)]
73. Jin, X.; Li, Z.; Yang, G.; Yang, H.; Feng, H.; Xu, X.; Wang, J.; Li, X.; Luo, J. Winter wheat yield estimation based on multi-source medium resolution optical and radar imaging data and the AquaCrop model using the particle swarm optimization algorithm. *ISPRS J. Photogramm. Remote Sens.* **2017**, *126*, 24–37. [[CrossRef](#)]
74. Krasileva, K.V.; Vasquez-Gross, H.A.; Howell, T.; Bailey, P.; Paraiso, F.; Clissold, L.; Simmonds, J.; Ramirez-Gonzalez, R.H.; Wang, X.; Borrill, P. Uncovering hidden variation in polyploid wheat. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 913–921. [[CrossRef](#)] [[PubMed](#)]
75. Wang, P.; Zou, M.; Liu, K.; Gu, Z.; Yang, R. Effect of mild thermal treatment on the polymerization behavior, conformation and viscoelasticity of wheat gliadin. *Food Chem.* **2018**, *239*, 984–992. [[CrossRef](#)]
76. Slade, A.J.; McGuire, C.; Loeffler, D.; Mullenberg, J.; Skinner, W.; Fazio, G.; Holm, A.; Brandt, K.M.; Steine, M.N.; Goodstal, J.F. Development of high amylose wheat through TILLING. *BMC Plant Biol.* **2012**, *12*, 10. [[CrossRef](#)]
77. Guo, H.; Yan, Z.; Li, X.; Xie, Y.; Xiong, H.; Liu, Y.; Zhao, L.; Gu, J.; Zhao, S.; Liu, L. Development of a High-Efficient Mutation Resource with Phenotypic Variation in Hexaploid Winter Wheat and Identification of Novel Alleles in the *TaAGPL-B1* Gene. *Front. Plant. Sci.* **2017**, *8*, 1404. [[CrossRef](#)]
78. Kim, H.; Yoon, M.-R.; Chun, A.; Tai, T.H. Identification of novel mutations in the rice starch branching enzyme I gene via TILLING by sequencing. *Euphytica* **2018**, *214*, 94. [[CrossRef](#)]

79. Hwang, J.E.; Jang, D.S.; Lee, K.J.; Ahn, J.W.; Kim, S.H.; Kang, S.Y.; Kim, D.S.; Kim, J.B. Identification of gamma ray irradiation-induced mutations in membrane transport genes in a rice population by TILLING. *Genes Genet. Syst.* **2017**, *91*, 245–256. [[CrossRef](#)]
80. Kusumi, K.; Hirotaka, S.; Kumamaru, T.; Iba, K. Increased leaf photosynthesis caused by elevated stomatal conductance in a rice mutant deficient in SLAC1, a guard cell anion channel protein. *J. Exp. Bot.* **2012**, *63*, 5635–5644. [[CrossRef](#)]
81. Caldwell, D.G.; McCallum, N.; Shaw, P.; Muehlbauer, G.J.; Marshall, D.F.; Waugh, R. A structured mutant population for forward and reverse genetics in Barley (*Hordeum vulgare* L.). *Plant. J.* **2004**, *40*, 143–150. [[CrossRef](#)]
82. Marzec, M.; Gruszka, D.; Tylec, P.; Szarejko, I. Identification and functional analysis of the *HvD14* gene involved in strigolactone signaling in *Hordeum vulgare*. *Physiol. Plant.* **2016**, *158*, 341–355. [[CrossRef](#)] [[PubMed](#)]
83. Bagge, M.; Xia, X.; Lübberstedt, T. Functional markers in wheat. *Curr. Opin. Plant. Biol.* **2007**, *10*, 211–216. [[CrossRef](#)] [[PubMed](#)]
84. Chawade, A.; Bräutigam, M.; Larsson, M.; Nakash, M.A.; Olsson, O.; Sikora, P.; Chen, T.; Vivekanand, V. Development and characterization of an oat TILLING-population and identification of mutations in lignin and β -glucan biosynthesis genes. *BMC Plant. Biol.* **2010**, *10*, 86. [[CrossRef](#)] [[PubMed](#)]
85. Till, B.J.; Reynolds, S.H.; Weil, C.; Springer, N.; Burtner, C.; Young, K.; Bowers, E.; Codomo, C.A.; Enns, L.C.; Odden, A.R. Discovery of induced point mutations in maize genes by TILLING. *BMC Plant. Biol.* **2004**, *4*, 12. [[CrossRef](#)]
86. Till, B.J.; Cooper, J.; Tai, T.H.; Colowit, P.; Greene, E.A.; Henikoff, S.; Comai, L. Discovery of chemically induced mutations in rice by TILLING. *BMC Plant. Biol.* **2007**, *7*, 19. [[CrossRef](#)] [[PubMed](#)]
87. Kim, S.-I.; Tai, T.H. Identification of novel rice low phytic acid mutations via TILLING by sequencing. *Mol. Breed.* **2014**, *34*, 1717–1729. [[CrossRef](#)]
88. Gottwald, S.; Bauer, P.; Komatsuda, T.; Lundqvist, U.; Stein, N. TILLING in the two-rowed barley cultivar ‘Barke’ reveals preferred sites of functional diversity in the gene *HvHox1*. *BMC Res. Notes* **2009**, *2*, 258. [[CrossRef](#)]
89. Sparla, F.; Falini, G.; Botticella, E.; Pirone, C.; Talamè, V.; Bovina, R.; Salvi, S.; Tuberosa, R.; Sestili, F.; Trost, P. New starch phenotypes produced by TILLING in barley. *PLoS ONE* **2014**, *9*, e107779. [[CrossRef](#)]
90. King, R.; Bird, N.; Ramirez-Gonzalez, R.; Coghill, J.A.; Patil, A.; Hassani-Pak, K.; Uauy, C.; Phillips, A.L. Mutation scanning in wheat by exon capture and next-generation sequencing. *PLoS ONE* **2015**, *10*, e0137549. [[CrossRef](#)]
91. Chen, A.; Dubcovsky, J. Wheat TILLING mutants show that the vernalization gene *VRN1* down-regulates the flowering repressor *VRN2* in leaves but is not essential for flowering. *PLoS Genet.* **2012**, *8*, e1003134. [[CrossRef](#)]
92. Uauy, C.; Paraiso, F.; Colasuonno, P.; Tran, R.K.; Tsai, H.; Berardi, S.; Comai, L.; Dubcovsky, J. A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant. Biol.* **2009**, *9*, 115. [[CrossRef](#)] [[PubMed](#)]
93. Colasuonno, P.; Incerti, O.; Lozito, M.L.; Simeone, R.; Gadaleta, A.; Blanco, A. DHPLC technology for high-throughput detection of mutations in a durum wheat TILLING population. *BMC Genet.* **2016**, *17*, 43. [[CrossRef](#)] [[PubMed](#)]
94. Rawat, N.; Sehgal, S.K.; Joshi, A.; Rothe, N.; Wilson, D.L.; McGraw, N.; Vadhani, P.V.; Li, W.; Gill, B.S. A diploid wheat TILLING resource for wheat functional genomics. *BMC Plant. Biol.* **2012**, *12*, 205. [[CrossRef](#)] [[PubMed](#)]
95. Wang, W.; Simmonds, J.; Pan, Q.; Davidson, D.; He, F.; Battal, A.; Akhunova, A.; Trick, H.N.; Uauy, C.; Akhunov, E. Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. *Theor. Appl. Genet.* **2018**, *131*, 2463–2475. [[CrossRef](#)]
96. Moehs, C.P.; Austill, W.J.; Holm, A.; Large, T.A.; Loeffler, D.; Mullenberg, J.; Schnable, P.S.; Skinner, W.; van Boxtel, J.; Wu, L. Development of reduced gluten wheat enabled by determination of the genetic basis of the *lys3a* low hordein barley mutant. *Plant. Physiol.* **2018**, *179*, 1692–1703. [[CrossRef](#)]

97. Li, W.; Guo, H.; Wang, Y.; Xie, Y.; Zhao, L.; Gu, J.; Zhao, S.; Zhao, B.; Wang, G.; Liu, L. Identification of novel alleles induced by EMS-mutagenesis in key genes of kernel hardness and starch biosynthesis in wheat by TILLING. *Genes Genom.* **2017**, *39*, 387–395. [[CrossRef](#)]
98. Severune, H.; Daniela, S.; Simon, G.K.; Bettina, K.; Thomas, W.; Gerhard, H.; Mirjam, N.F.; James, B.; Thomas, P.; Milena, O.; et al. The maize disease resistance gene Htn1 against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 8780–8785.
99. Stolarek, M.; Gruszka, D.; Braszewska-Zalewska, A.; Maluszynski, M. Alleles of newly identified barley gene *HvPARP3* exhibit changes in efficiency of DNA repair. *DNA Repair* **2015**, *28*, 116–130. [[CrossRef](#)]
100. Mendiondo, G.M.; Gibbs, D.J.; Szurman-Zubrzycka, M.; Korn, A.; Marquez, J.; Szarejko, I.; Maluszynski, M.; King, J.; Axcell, B.; Smart, K. Enhanced waterlogging tolerance in barley by manipulation of expression of the N-end rule pathway E3 ligase PROTEOLYSIS 6. *Plant. Biotechnol. J.* **2016**, *14*, 40–50. [[CrossRef](#)]
101. Hu, X. TILLING-based analysis of disease resistance genes in barley. *J. Shandong Agric. Univ.* **2012**.
102. Van Nimwegen, K.J.; van Soest, R.A.; Veltman, J.A.; Nelen, M.R.; van der Wilt, G.J.; Vissers, L.E.; Grutters, J.P. Is the \$1000 genome as near as we think? A cost analysis of next-generation sequencing. *Clin. Chem.* **2016**, *62*, 1458–1464. [[CrossRef](#)] [[PubMed](#)]
103. Wang, S.; Wong, D.; Forrest, K.; Allen, A.; Chao, S.; Huang, B.E.; Maccaferri, M.; Salvi, S.; Milner, S.G.; Cattivelli, L. Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. *Plant. Biotechnol. J.* **2014**, *12*, 787–796. [[CrossRef](#)] [[PubMed](#)]
104. Rothberg, J.M.; Hinz, W.; Rearick, T.M.; Schultz, J.; Mileski, W.; Davey, M.; Leamon, J.H.; Johnson, K.; Milgrew, M.J.; Edwards, M. An integrated semiconductor device enabling non-optical genome sequencing. *Nature* **2011**, *475*, 348. [[CrossRef](#)] [[PubMed](#)]
105. Wetterstrand, K.A. DNA sequencing costs: Data from the NHGRI Genome Sequencing Program (GSP). *Nat. Human Res. Instit.* **2013**.
106. Rigola, D.; van Oeveren, J.; Janssen, A.; Bonné, A.; Schneiders, H.; van der Poel, H.J.; van Orsouw, N.J.; Hogers, R.C.; de Both, M.T.; van Eijk, M.J. High-throughput detection of induced mutations and natural variation using KeyPoint™ technology. *PLoS ONE* **2009**, *4*, e4761. [[CrossRef](#)]
107. Tasai, H.; Tyson, H.; Rebecca, N.; Victor, M.; Brian, W.; Kathie, N.; Meric, N.; Joseph, F.; Cristobal, U. Discovery of rare mutations in populations: TILLING by sequencing. *Plant. Physiol.* **2011**. [[CrossRef](#)]
108. Henry, I.M.; Nagalakshmi, U.; Lieberman, M.C.; Ngo, K.J.; Krasileva, K.V.; Vasquez-Gross, H.; Akhunova, A.; Akhunov, E.; Dubcovsky, J.; Tai, T.H. Efficient genome-wide detection and cataloging of EMS-induced mutations using exome capture and next-generation sequencing. *Plant. Cell* **2014**, *26*, 1382–1397. [[CrossRef](#)]
109. Reddy, T.V.; Dwivedi, S.; Sharma, N.K. Development of TILLING by sequencing platform towards enhanced leaf yield in tobacco. *Ind. Crops Prod.* **2012**, *40*, 324–335. [[CrossRef](#)]
110. Krothapalli, K.; Buescher, E.M.; Li, X.; Brown, E.; Chapple, C.; Dilkes, B.P.; Tuinstra, M.R. Forward genetics by genome sequencing reveals that rapid cyanide release deters insect herbivory of *Sorghum bicolor*. *Genetics* **2013**, *195*, 309–318. [[CrossRef](#)]
111. Elahi, N.; Duncan, R.W.; Stasolla, C. Decreased seed oil production in *FUSCA3* *Brassica napus* mutant plants. *Plant. Physiol. Biochem.* **2015**, *96*, 222–230. [[CrossRef](#)]
112. Minoia, S.; Boualem, A.; Marcel, F.; Troadec, C.; Quemener, B.; Cellini, F.; Petrozza, A.; Vigouroux, J.; Lahaye, M.; Carriero, F. Induced mutations in tomato *SExp1* alter cell wall metabolism and delay fruit softening. *Plant. Sci.* **2016**, *242*, 195–202. [[CrossRef](#)] [[PubMed](#)]
113. Ma, X.; Zhu, Q.; Chen, Y.; Liu, Y.-G. CRISPR/Cas9 platforms for genome editing in plants: Developments and applications. *Mol. Plant.* **2016**, *9*, 961–974. [[CrossRef](#)]
114. Petolino, J.F.; Srivastava, V.; Daniell, H. Editing Plant Genomes: A new era of crop improvement. *Plant. Biotechnol. J.* **2016**, *14*, 435–436. [[CrossRef](#)] [[PubMed](#)]
115. Shan, Q.; Wang, Y.; Li, J.; Gao, C. Genome editing in rice and wheat using the CRISPR/Cas system. *Nat. Protoc.* **2014**, *9*, 2395. [[CrossRef](#)] [[PubMed](#)]
116. Zhang, H.; Xu, R.; Xie, C.; Huang, C.; Liao, H.; Xu, Y.; Wang, J.; Li, W.-X. Large-scale evaluation of maize germplasm for low-phosphorus tolerance. *PLoS ONE* **2015**, *10*, e0124212. [[CrossRef](#)]
117. Xu, Y.; Li, P.; Zou, C.; Lu, Y.; Xie, C.; Zhang, X.; Prasanna, B.M.; Olsen, M.S. Enhancing genetic gain in the era of molecular breeding. *J. Exp. Bot.* **2017**, *68*, 2641–2666. [[CrossRef](#)]

118. Jiang, W.; Zhou, H.; Bi, H.; Fromm, M.; Yang, B.; Weeks, D.P. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Res.* **2013**, *41*, e188. [[CrossRef](#)]
119. Wang, W.; Pan, Q.; He, F.; Akhunova, A.; Chao, S.; Trick, H.; Akhunov, E. Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. *CRISPR J.* **2018**, *1*, 65–74. [[CrossRef](#)]
120. Avni, R.; Nave, M.; Barad, O.; Baruch, K.; Twardziok, S.O.; Gundlach, H.; Hale, I.; Mascher, M.; Spannagl, M.; Wiebe, K. Wild emmer genome architecture and diversity elucidate wheat evolution and domestication. *Science* **2017**, *357*, 83–97. [[CrossRef](#)]

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