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TILLING - a shortcut in functional genomics

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Abstract Recent advances in large-scale genome sequencing projects have opened up new possibilities for the application of conventional mutation techniques in not only forward but also reverse genetics strategies. TILLING (Targeting Induced Local Lesions IN Genomes) was developed a decade ago as an alternative to insertional mutagenesis. It takes advantage of classical mutagenesis, sequence availability and high-throughput screening for nucleotide polymorphisms in a targeted sequence. The main advantage of TILLING as a reverse genetics strategy is that it can be applied to any species, regardless of its genome size and ploidy level. The TILLING protocol provides a high frequency of point mutations distributed randomly in the genome. The great mutagenic potential of chemical agents to generate a high rate of nucleotide substitutions has been proven by the high density of mutations reported for TILLING populations in various plant species. For most of them, the analysis of several genes revealed 1 mutation/200–500 kb screened and much higher densities were observed for polyploid species, such as wheat. High-throughput TILLING permits the rapid and low-cost discovery of new alleles that are induced in plants. Several research centres have established a TILLING public service for various plant species. The recent trends in TILLING procedures rely on the diversification of bioinformatic tools, new methods of mutation detection, including mismatch-specific and sensitive endonucleases, but also various alternatives for LI-COR screening and single nucleotide polymorphism (SNP) discovery using next-generation sequencing technologies. The TILLING strategy has found numerous applications in functional genomics. Additionally, wide applications of this throughput method in basic and applied research have already been implemented through modifications of the original TILLING strategy, such as Ecotilling or Deletion TILLING.

Keywords TILLING platform · Plant mutagenesis · Bioinformatic tools · Reverse genetics · Next-generation sequencing technologies

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Introduction

One of the most direct ways of establishing gene function is to identify a mutation in the specific gene and to link this mutation to the phenotypic change in the mutated organism. In the forward genetics approach ("from mutation through phenotype to the gene"), large mutated populations have been created and screened for alterations in the trait or biological process of interest. Over the decades, large mutant collections have been developed for many model organisms. These isolated mutants have then served for the identification of the genes underlying the change in phenotype. The sequence of the gene responsible for the altered phenotype can be isolated using the process of map-based cloning. Although this approach is both time-consuming and labour-intensive, it has been successfully applied for cloning several genes, even in species with large genomes, such as barley and wheat (Keller et al. 2005; Komatsuda et al. 2007; Krattinger et al. 2009; Zhang et al. 2009).

Recent advances in large-scale genome sequencing projects have opened up new possibilities for the application of mutation techniques in basic studies and in the improvement of crops. The reverse genetics strategy ("from

gene sequence to phenotype") has widely replaced the forward approach in studies involved in detecting gene function. This strategy is based on the alteration of a gene structure or its activity, followed by an analysis of the associated change in plant phenotype. Several reverse genetics technologies, such as insertional mutagenesis with T-DNA, transposon/retrotransposon tagging or gene silencing using RNA interference, have been proposed for plant functional genomics (for reviews, see: Alonso and Ecker 2006; Small 2007; Boutros and Ahringer 2008; Hirochika 2010; Bolle et al. 2011; Upadhyaya et al. 2011). However, the majority of these methods are fully applicable only for model plants with small genomes, such as Arabidopsis or rice, and even in these species, there are some drawbacks that limit their utilisation.

TILLING (Targeting Induced Local Lesions IN Genomes) was developed a decade ago as an alternative to insertional mutagenesis in *Arabidopsis thaliana* (McCallum et al. 2000). TILLING takes advantage of classical mutagenesis, sequence availability and high-throughput screening for nucleotide polymorphisms in a targeted sequence. It combines the high frequency of mutations induced by traditional mutagenesis with sensitive techniques for discovering single nucleotide mutations. The main advantage of TILLING as a reverse genetics strategy is that it can be applied to any plant species, regardless of its genome size, ploidy level or method of propagation. Chemical mutagens, which are usually used in TILLING protocols, provide a high frequency of point mutations distributed randomly in the genome. An analysis of mutations induced by ethyl methanesulphonate (EMS) in 192 Arabidopsis genes revealed about ten mutations per gene among the 3,000 M_2 plants examined (Greene et al. 2003). It was estimated that each M_2 plant carried, on average, 720 mutations (Till et al. 2003), while only 1.5 T-DNA insertions per mutant line were detected in the Arabidopsis insertion populations (Alonso et al. 2003). Thus, much smaller populations are required to reach saturation mutagenesis using TILLING—ca. 5,000 M_1 plants in Arabidopsis (Østergaard and Yanofsky 2004) as compared to 360,000 lines in T-DNA mutagenesis (Alonso and Ecker 2006). The application of TILLING makes the functional analysis of large genomes as well as small genes, which are difficult targets for insertional mutagenesis, possible.

Another great advantage of TILLING technology relies on the ability of chemical mutagens to create a spectrum of mutations, including missense changes, truncation and mutations in splice junction sequences. In contrast to insertional mutagenesis that generates mostly gene knock-outs, using TILLING, it is possible to induce a series of alleles in a targeted locus. In addition to loss-of-function alleles, chemical mutagens generate gain-of-function and hypomorphic alleles that can provide a range of phenotypes (Alonso and Ecker 2006). The mutations are stable, which is not always the case for alternative methods of reverse genetics utilising RNAi silencing or transposon, e.g. Ac/Ds tagging. In addition, RNAi technology and insertional mutagenesis through T-DNA or transposon tagging relies on genetic transformation. TILLING does not require transformation and, thus, is the only reverse genetics strategy applicable for species that are not transformable or recalcitrant. It is recommended as non-GMO technology, so when using TILLING, GMO procedures and controversies are avoided. Moreover, TILLING is not technically demanding and can be performed at a relatively low cost.

The TILLING strategy was initially developed as a discovery platform for functional genomics, but it soon became a valuable tool in crop breeding as an alternative to the transgenic approach. The feasibility of TILLING has already been demonstrated for a large number of agronomically important crops, including rice, barley, wheat, maize, sorghum, soybean, rapeseed and tomato plants (Table 1). Large-scale TILLING services have also been created for model animal organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio* and *Rattus norvegicus* (Winkler et al. 2005; Gilchrist et al. 2006a; Wienholds et al. 2003; Smits et al. 2004, respectively).

The general protocol for the creation of a TILLING platform in plants includes the following steps (Fig.1):

1. Creation of mutated populations
   - Chemical mutagenesis
   - Development of M_1 and M_2 generations
   - DNA extraction from individual M_2 plants
   - Creation of DNA pools of 5–8 M_2 plants
   - Setting up an M_3 seed bank
2. Detection of mutations in a targeted sequence
   - Polymerase chain reaction (PCR) amplification of the targeted DNA segment using pooled DNA as a template
   - Detection of mutations using different procedures, e.g. cleavage by specific endonuclease, denaturing high-performance liquid chromatography (DHPLC) or high-throughput sequencing
   - Identification of the individual M_2 plant carrying the mutation
   - Sequencing the target gene segment to confirm the mutation and to determine the type of nucleotide change
3. Analysis of the mutant phenotype

A mutated population becomes a TILLING platform when the DNA samples and seeds collected from a large M_2 population are archived and put into databases. Usually,

Table 1 Description of TILLING populations developed in model and crop plants

Species/ploidy level	Cultivar/line/ ecotype	Mutagen	Size of M_2 population	No. of genes analysed	Total sequence screened (Mb)	Total no. of mutations detected	Mutation density (1mutation/kb)	Reference
Arabidopsis thaliana (2x)	Columbia-0	EMS	n.a.	192	576.0	1,890	300	Greene et al. 2003
	Columbia-0	EMS	6,912	100	301.6	1,774	170*	Till et al. 2003
	Landsberg erecta	EMS	3,712	14	40.0	450	89*	Martín et al. 2009
Avena sativa (6x)	Bekinda	EMS	2,600	2	0.53	16	33	Chawade et al. 2010
Brassica oleracea (2x)	TO1000	EMS	2,263	15	11.2	25	447*	Himelblau et al. 2009
Brassica rapa (2x)	R-o-19	EMS	9,216	6	32.8	617	60	Stephenson et al. 2010
Cucumis melo (2x)	CharMono	EMS	4,023	11	65.1	134	573	Dahmani-Mardas et al. 2010
Glycine max (4x)	Forrest	EMS	529	7	5.3	32	140*	Cooper et al. 2008
	Williams 82	EMS	768	7	7.7	12	550*	Cooper et al. 2008
	Williams 82	MNU	768	7	7.7	47	140*	Cooper et al. 2008
Hordeum vulgare (2x)	Optic	EMS	9,216	2	12.3	10	1,000	Caldwell et al. 2004
	Morex	NaN3	4,906	4	10.2	22	374*	Talamè et al. 2008
	Barke	EMS	10,279	6	52.3	81	500*	Gottwald et al. 2009
	Lux	NaN3	9,575	2	12.3	5	2,500	Lababidi et al. 2009
	Sebastian	NaN_3-MNU	10,000	12	41.0	174	235	Unpublished data
	DH 930-36	MNU	1,372	2	4.4	9	486	Unpublished data
	DH 930-36	Gamma rays	1,753	1	3.3	1	3,297	Unpublished data
Lotus japonicus (2x)	B-129 Gifu	EMS	4,904	61	288.9	576	502	Perry et al. 2009
Oryza sativa (2x)	Nipponbare	EMS	768	10	8.0	27	294*	Till et al. 2007
	Nipponbare	NaN_3-MNU	768	10	8.0	30	265*	Till et al. 2007
	Taichung 65	MNU	767	3	3.2	24	135	Suzuki et al. 2008
Pisum sativum (2x)	n.a.	EMS	4,717	1	9.7	50	193	Triques et al. 2008
Solanum lycopersicum (2x)	Tpaadasu	EMS	8,225	5	32.8	44	737	Gady et al. 2009
	Red Setter	EMS	4,741	7	36.6	25	322	Minoia et al. 2010
	Red Setter	EMS	1,926	7	12.5	41	574	Minoia et al. 2010
	M82	EMS	4,759	19	146.9	256	574	Piron et al. 2010
Sorghum bicolor (2x)	BTx623	EMS	1,600	4	6.5	5	526	Xin et al. 2008
Triticum aestivum (6x)	Express	EMS	10,000	2	4.0	196	24	Slade et al. 2005
	Hard Red Spring	EMS	1,536	3	11.5	186	38*	Uauy et al. 2009
	Chara	HII	20,000	1	24.7	294	84	Fitzgerald et al. 2010
Triticum durum (4x)	Kronos	EMS	8,000	2	1.3	50	40	Slade et al. 2005
	Kronos	EMS	1,386	2	5.6	93	51*	Uauy et al. 2009
Zea mays (2x)	B73	EMS	750	11	12.8	17	485	Till et al. 2004a, b

*100–200 bp of each amplicon were excluded from the calculation of the total sequence length used for estimation of mutation density

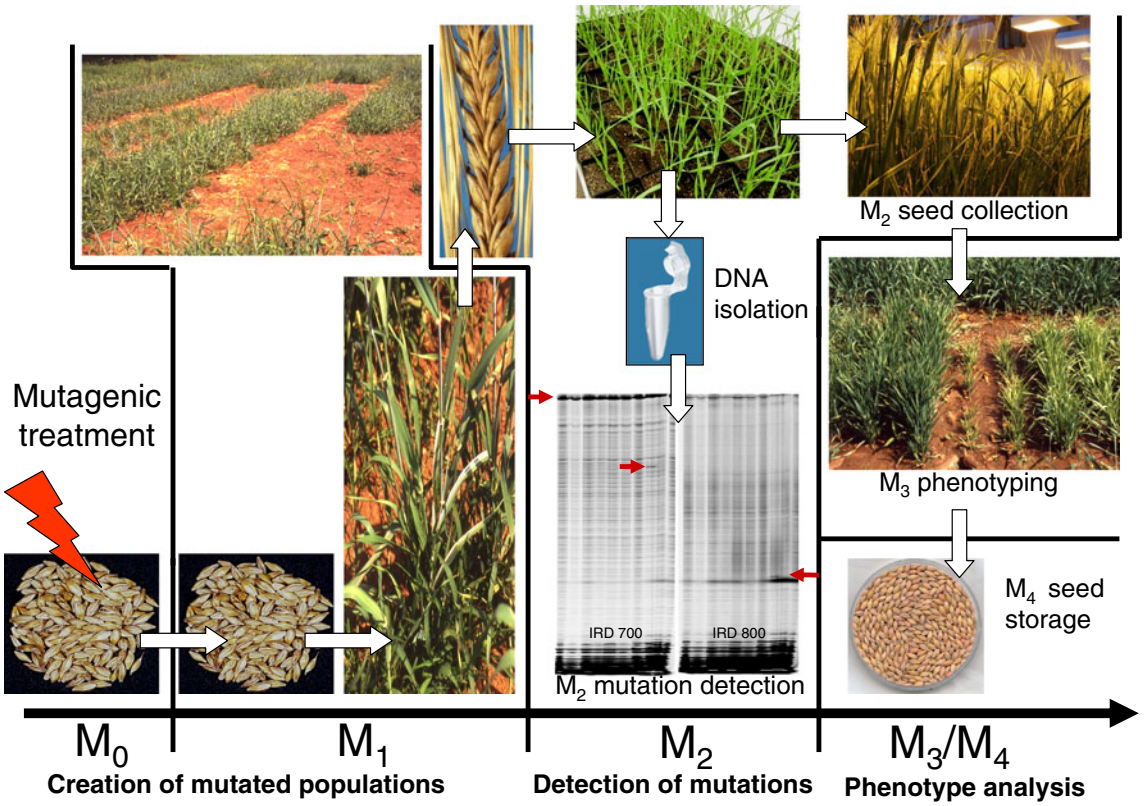

Fig. 1 Development of a TILLING platform in barley

platforms of 3,000–5,000 M_2 individuals are created, although larger populations that include 10,000 plants have also been reported. Almost all TILLING populations were developed using chemical mutagens, among them, the alkylating agent (EMS) was most often applied. The great mutagenic potential of chemical agents has been proven by the high density of mutations reported for established TILLING populations (Tables 1 and 2).

Once established, the TILLING platform can serve as a permanent source of mutations for both forward and reverse genetics. In many cases, phenotypic observations of the M_2 generation are performed and large M_3 generations are generated for forward screening (Talamè et al. 2008; Wang et al. 2008a; Himelblau et al. 2009; Minoia et al. 2010). A detailed phenotypic evaluation of M_2 and M_3 progeny makes it possible to enrich the population that is screened for mutations in a specific gene, or for plants bearing a phenotypic change related to the studied metabolic or developmental process. Such an approach was successfully demonstrated by Perry et al. (2003) for the analysis of genes controlling the root symbiosis of *Lotus japonicus* using a TILLING population that was pre-selected for root nodulation mutants. Additionally, large M_3 generations can provide a resource for the direct forward selection of mutants tolerant/hypersensitive to a range of abiotic and biotic stresses.

To facilitate the systematic gathering of information about the developed platform, including molecular and phenotypic data, TILLING databases are created and often made publicly available. Examples of such databases are presented in the further part of this paper, followed by a description of TILLING services, bioinformatic tools and methods of mutation detection, including the application of new generation technologies and modifications of the TILLING strategy. Presented below are also examples of the use of this technology in the creation of useful new alleles in many crop species, which are alternatives to the transgenic approach in plant breeding.

TILLING platforms and services

Creating a TILLING population requires that a lot of information to be collected in a relevant database. Most of the available databases contain data on 5,000 to 13,000 plants of the M_2 population. It is necessary to keep a description of every plant phenotype, information on the availability of the DNA extracted from each plant and seeds in the seed bank in such a database and to systematically gather information about the molecular and genetic research that is being carried out. Furthermore, this data has to be saved properly, in order to allow the results to be searched

Table 2 Functional mutation spectrum and types of nucleotide substitutions detected in different TILLING populations

Mutagen	Species	No. of genes analysed	Total no. of mutations detected	Missense (%)	Silent (%)	Nonsense (%)	Non-coding (%)	Transitions G/C > A/T (%)	Transitions A/T > G/C (%)	Transversions (%)	Reference
EMS	*Arabidopsis thaliana*	192	1,890	50	45	5	0	99	n.a.	n.a.	Greene et al. 2003
		100	1,774/1,063*	49.7	45.8	4.5	0	98	n.a.	n.a.	Till et al. 2003
		14	450	38.2	36.5	4.2#	21.1	n.a.	n.a.	n.a.	Martín et al. 2009
	Avena sativa	2	16	50	50	0	0	94.4	0	5.6	Chawade et al. 2010
	Brassica napus	1	19	63.2	36.8	0	0	94.7	0	5.3	Wang et al. 2008a, b
	Brassica oleracea	15	25	60	40	0	n.a.	100	0	0	Himelblau et al. 2009
	Brassica rapa	6	617/306*	63.4	34.3	2.3	n.a.	n.a.	n.a.	n.a.	Stephenson et al. 2010
	Cucumis melo	11	134	65.1	31.3	2.4	n.a.	97.8	0	2.2	Dahmani-Mardas et al. 2010
	Glycine max	7	32	66	34	0	n.a.	93.8	0	6.2	Cooper et al. 2008
		7	12	33	58	8	n.a.	75	0	16.7	Cooper et al. 2008
		7	25	44	52	4	n.a.	92	4	4	Cooper et al. 2008
	Hordeum vulgare	2	10	60	40	0	0	70	10	20	Caldwell et al. 2004
		6	81	35.8	39.5	3.7#	21	n.a.	n.a.	n.a.	Gottwald et al. 2009
	Lotus japonicus	61	576	47.7	20.5	2.8	29	93.4	n.a.	n.a.	Perry et al. 2009
	Oryza sativa	10	27	48.1	33.4	18.5	n.a.	70.4	0	29.6	Till et al. 2007
	Pisum sativum	1	50	60	34	0	6				Triques et al. 2008
	Solanum lycopersicum (2x)	7	66	62.4	37.6	0	n.a.	44.3	0	55.7	Minoia et al. 2010
		19	256	33.2	20.7	2.7	43.4	n.a.	n.a.	n.a.	Piron et al. 2010
	Triticum aestivum	2	196	34.2	n.a.	1.0	n.a.	99.3	0	0.7	Slade et al. 2005
		2	140	45.4	30.3	9.2	15.1	99.2	0	0.8	Dong et al. 2009
		3	13	46	38.6	7.7	n.a.	n.a.	n.a.	n.a.	Sestili et al. 2010
	Triticum durum	2	50	34	n.a.	2.0	n.a.	100	0	0	Slade et al. 2005
	Zea mays	11	17	58.8	41.2	0	0	100	0	0	Till et al. 2004a
	Medicago truncatula	56	512	67.4	27.7	4.9	n.a.	98.8	0	1.2	Le Signor et al. 2009
		10	34	58.8	38.2	3.0	n.a.	n.a.	n.a.	n.a.	Le Signor et al. 2009
MNU	*Glycine max*	7	47	45	51	4	n.a.	89.4	0	10.6	Cooper et al. 2008
	Oryza sativa	3	24	54.2	41.7	0	4.1	91.7	0	8.3	Suzuki et al. 2008
	Hordeum vulgare	2	9	55.6	22.2	0	22.2	55.5	0	44.5	Unpublished data
NaN_3	*Hordeum vulgare*	4	22	68.2	18.2	0	13.6	95.5	0	4.5	Talamè et al. 2008
		2	5	60	0	0	40	80	0	20	Lababidi et al. 2009
NaN_3/MNU	*Hordeum vulgare*	13	180	44.4	36.1	1.7	17.8	89.4	1.1	6.2	Unpublished data
	Oryza sativa	10	30	56.7	33.3	0	10	66.7	0	33.3	Till et al. 2007
Gamma rays	*Oryza sativa*	25	6	33.3	0	0	66.7	n.a.	n.a.	50	Sato et al. 2006
	Hordeum vulgare	1	1	0	100	0	0	0	0	100	Unpublished data

*The number of mutations for which the analysis of mutation types were proceed

Truncation include mutations generating premature stop codons and mutations in splice sites

and sorted. That is why creating a database containing all of this information about the investigated plants is a very important part of every project involving the development of a TILLING platform. It is also desirable that such a database will be publicly available on the Internet. Most analyses of a phenotype apply to all stages of plant development and are divisible into very specific categories and subcategories. Typically, the main categories describe the attributes of the whole plant (e.g. time of flowering, size/height of plant, plant architecture) or individual plant organs (e.g. seeds, fruits, tillers, spikes), whereas subcategories apply to the phenotypes that have already been observed (e.g. leaf size, branching, abnormal spikes). The databases that are accessible on the Internet contain 48 to 107 subcategories grouped under various major categories. Additionally, descriptions of plants showing phenotypic changes have been supplied with photographic documentation. Some of the databases also contain information about genes that have already been analysed and any identified mutations in their sequences. In this case, the analysed sequence is entered into database with the identified mutations indicated and with information about its localisation in the exon or intron. Additionally, there is information about the nature of the mutation and its impact on the amino acid sequence and protein structure, if available.

Some publicly accessible databases may constitute independent websites, whose only purpose is to describe either the specific project (LycoTILL, http://www.agrobios.it/tilling) or several projects carried out by one team (UTILLdb, http://urgv.evry.inra.fr/UTILLdb). When creating meta-services involving a number of different databases that already exist on various aspects of studies on one organism, TILLING databases can also be included (MaizeGBD, http://www.maizegdb.org/). A common feature of all of the accessible TILLING databases is the information about the phenotype of the analysed plants. Teams that previously only shared information about identified mutations now update the databases with the results of phenotyping, including photos and a detailed description of any changes in the phenotype (e.g. the Maize TILLING Project). There are only a few databases related to TILLING projects that are accessible. These databases have been created at different times and by different teams; therefore. they differ greatly in their content and the available options for users (Table 3).

High-throughput TILLING permits the rapid and low-cost discovery of new induced alleles in plants. However, the creation of a large TILLING platform is a time-consuming, labour-intensive and expensive task. Several research centres from Europe and North America have established TILLING public services for various plant species: *Medicago truncatula*, *L. japonicus* and *Brassica rapa* at the John Innes Centre (RevGenUK); *Pisum sativum* and *Solanum lycopersicum* at the French National Institute for Agriculture Research (INRA) (UTILLdb); *Oryza sativa*, *S. lycopersicum* and *A. thaliana* at the University of California; *A. thaliana* by the Seattle TILLING Project (STP); *Zea mays* at Purdue University; *Glycine max* at Southern Illinois University; *Avena sativa* at CropTailor AB; *A. thaliana*, *B. oleracea* and *B. napus* at the University of British Columbia (CAN-TILL); *Hordeum vulgare* at the Department of Agro-Environmental Sciences and Technology (DiSTA) of the University of Bologna (TILLMore: a TILLING resource in Barley Morex). Information about the assayed genes (TILLed genes) using the TILLING strategy is not always available on the websites because this information could be of commercial value. It is possible to order the TILLING analysis of any known gene that is involved in a process that is being investigated or a trait in particular species (Table 3). However, it is important to remember that knowing the genomic sequence of the chosen gene or genes is necessary and the user must decide whether the gene is a good candidate for TILLING. On the websites of the above-mentioned public services, the potential user can find all of the information which is necessary to start a project, e.g. how to find the best region of the gene to target and how design the proper primers using free web-based tools. Screening the selected region of the requested gene for mutations discovery is usually provided by the service. After analysis, the service provides the user with a list of mutations in the investigated sequence(s) and seeds of related mutants. In a short time, the scientists and plant breeders can take advantage of existing sources of the induced genetic diversity in those plant species for which commercial services are available. Several research centres have created TILLING populations for particular species and cultivars that are of interest. The results of these investigations can be found in related publications (Table 4).

Mutagens

Chemical mutagenesis causes both point mutations, which are irreversible and produced in relatively high densities, and also chromosome breaks that cause various chromosomal rearrangements, which can reduce fertility and affect lethality. Unlike insertional mutagenesis, the high density of chemically induced point mutations makes TILLING suitable for targeting small genes or single protein domains that are encoded by large genes (Till et al. 2003). In contrast to insertional mutagenesis, the TILLING strategy is general, because chemical mutagenesis has been successfully applied to most major taxa (Henikoff and Comai 2003). Alkylating agents are the most commonly applied chemicals in plant mutagenesis. They constitute a class of base-modifying compounds which directly alter the structure and properties of the bases. DNA

Table 3 TILLING public platforms and commercial services for different plant species

Name of database	Species	Phenotype description	Information about TILLed genes*	Commercial service	Link	Reference
Tomato Mutant Database	*Solanum lycopersicum*	+	+	−	http://zamir.sgn.cornell.edu/mutants/	Menda et al. 2004
Lotus japonicus mutant finder	*Lotus japonicus*	+	+	−	http://data.jic.bbsrc.ac.uk/cgi-bin/lotusjaponicus/	Perry et al. 2003
UTILLdb	*Pisum sativumSolanum lycopersicumBrachypodium distachyon*	+	+	+	http://urgv.evry.inra.fr/UTILLdb	Dalmais et al. 2008
LycoTILL	*Solanum lycopersicum*	+	−	−	http://www.agrobios.it/tilling/	Minoia et al. 2010
Soybean Mutation Database	*Glycine max*	+	+	+	http://www.soybeantilling.org/	–
RevGenUK	*Medicago truncatulaLotus japonicusBrassica rapa*	−	−	+	http://revgenuk.jic.ac.uk/about.htm	Stephenson et al. 2010
UC Davis TILLING Core	*Oryza sativaSolanum lycopersicumArabidopsis thaliana*	−	−	+	http://tilling.ucdavis.edu/index.php/Main_Page	Till et al. 2007
Seattle TILLING Project	*Arabidopsis thaliana*	−	−	+	http://tilling.fhcrc.org/	–
NordGen, CropTailor AB	*Avena sativa*	−	−	+	http://www.nordgen.org, http://www.croptailor.com	Chawade et al. 2010
CAN-TILL	*Arabidopsis thalianaBrassica oleraceaBrassica napus*	−	−	+	http://www.botany.ubc.ca/can-till/	Himelblau et al. 2009
TILLMore	*Hordeum vulgare*	+	−	+	http://www.distagenomics.unibo.it/TILLMore/	Talamè et al. 2008

*TILLed gene—gene analysed with the use of the TILLING strategy

Table 4 TILLING populations of different plant species for which the databases and/or commercial services are not available on the Internet at present

Species	Centre	Country	Reference
Arabidopsis thaliana	Fred Hutchinson Cancer Research Center	USA	Greene et al. 2003
	Cereon Genomics	USA	Jander et al. 2003
	Fred Hutchinson Cancer Research Center	USA	Till et al. 2003
	Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología (CNB)	Spain	Martín et al. 2009
Avena sativa	Department of Plant and Environmental Sciences, Goteborg University	Sweden	Chawade et al. 2010
Brassica oleracea	Department of Biology, California Polytechnic State University	USA	Himelblau et al. 2009
Brassica rapa	Department of Crop Genetics, John Innes Centre	UK	Stephenson et al. 2010
Cucumis melo	Unite de Recherche en Genomique Vegetale, INRA-UEVE-CNRS	France	Dahmani-Mardas et al. 2010
Glycine max	Fred Hutchinson Cancer Research Center	USA	Cooper et al. 2008
Hordeum vulgare	Scottish Crop Research Institute	UK	Caldwell et al. 2004
	Department of Agroenvironmental Sciences and Technology, University of Bologna	Italy	Talamè et al. 2008
	Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)	Germany	Gottwald et al. 2009
	Department of Agriculture and Ecology, University of Copenhagen	Denmark	Lababidi et al. 2009
Lotus japonicus	The Sainsbury Laboratory	UK	Perry et al. 2009
	Metabolic Biology, John Innes Centre	UK	Vriet et al. 2010
Medicago truncatula	Department of Disease and Stress Biology, John Innes Centre	UK	Rogers et al. 2009
Oryza sativa	Graduate School of Agricultural Science, Tohoku University	Japan	Sato et al. 2006
	Fred Hutchinson Cancer Research Center	USA	Till et al. 2007
	Genetic Strains Research Center	Japan	Suzuki et al. 2008
Solanum lycopersicum	Wageningen UR, Plant Breeding, Wageningen University and Research Center	The Netherlands	Gady et al. 2009
	Metapontum Agrobios	Italy	Minoia et al. 2010
	Unité de Recherche en Génomique Végétale, UMR INRA-CNRS-Uni	France	Piron et al. 2010
Sorghum bicolor	Plant Stress and Germplasm Development Unit	USA	Xin et al. 2008
Triticum aestivum	Anawah Inc.	USA	Slade et al. 2005
	Plant Breeding Institute	Australia	Dong et al. 2009
	Department of Plant Sciences, University of California	USA	Uauy et al. 2009
	CSIRO Plant Industry	Australia	Fitzgerald et al. 2010
	Department of Agrobiology and Agrochemistry, University of Tuscia	Italy	Sestili et al. 2010
Triticum turgidum	Anawah Inc.	USA	Slade et al. 2005
	Department of Plant Sciences, University of California	USA	Uauy et al. 2009
Zea mays	Basic Sciences Division, Fred Hutchinson Cancer Research Center	USA	Till et al. 2004a

bases differ in their vulnerability to chemical modification by alkylating mutagens: ring nitrogens are more reactive than oxygens and the N^7 position of the guanine and N^3 position of the adenine are the most prone to modification. Alkylating agents cause every type of point mutation: transitions, transversions, deletions and frameshifts, as well as chromosome breakages (Maluszynski et al. 2003).

In the majority of TILLING experiments, especially those conducted on Arabidopsis, EMS has been applied as a mutagen. In addition to this species, EMS has been used as the mutagen in TILLING experiments performed on *B. napus*, *B. oleracea*, *G. max*, *H. vulgare*, *L. japonicus*, *M. truncatula*, *O. sativa*, *Sorghum bicolor*, *Triticum aestivum*, *T. durum* and *Z. mays* (Gilchrist and Haughn 2005; Martín et al. 2009). EMS-induced mutations are randomly distributed in the genome and a high degree of mutational saturation can be achieved without excessive DNA damage (Gilchrist and Haughn 2005). In Arabidopsis, 5% of the mutations induced by this mutagen in coding regions result in the premature termination of the gene product (nonsense mutations) or mutations in splice sites, whereas ca. 50% lead to missense mutations (Greene et al. 2003; Martín et al. 2009). EMS alkylates guanine bases and leads to mispairing: alkylated guanine pairs with thymine, which results mainly in G/C to A/T transitions (Henikoff and Comai 2003). This type of transition makes up more than 99% of all EMS-induced mutations in Arabidopsis, maize and wheat. However, when the frequencies of various types

of EMS-induced mutations were analysed in other species (tomato, rice and barley) G/C to A/T transitions constituted no more than 70% (Minoia et al. 2010). The discussed mutation percentages and frequencies are reported in Tables 1 and 2.

Another alkylating agent, *N*-methyl-*N*-nitrosourea (MNU, MNH), induced only G/C to A/T transitions in several TILLING experiments in *G. max* and *O. sativa* (Cooper et al. 2008 and Suzuki et al. 2008, respectively). The mutation frequency induced by this mutagen in *G. max* was comparable to the frequency induced in this species by EMS (1 mutation/140 kb) (Cooper et al. 2008). However, in rice, MNU-induced mutations had a frequency that was two times higher (1 mutation/135 kb) than EMS (1 mutation/300 kb) (Till et al. 2007; Suzuki et al. 2008; Martín et al. 2009). However, it should be kept in mind that, in the case of the MNU treatment, the panicles were exposed to the mutagen, while in the EMS experiment, the seeds were treated with the mutagen. Another confirmation that the treatment procedure is crucial for the mutation frequency obtained is the comparison of mutation frequencies between two TILLING experiments in rice, both using MNU. In one of these experiments, rice panicles were treated with 1 mM MNU (Suzuki et al. 2008), whereas in the second, the rice seeds were exposed to a combined treatment with 1 mM of sodium azide and 15 mM MNU (Till et al. 2007). The mutation frequency obtained after the treatment of the panicles was higher (1 mutation/135 kb) than the frequency which resulted from the combined treatment of the seeds (1 mutation/265 kb) (Suzuki et al. 2008 and Till et al. 2007, respectively).

Sodium azide, which has been used as the mutagen in several experiments, is a chemical compound that becomes mutagenic only in some organisms, e.g. *Escherichia coli*, *Saccharomyces cerevisiae*, *H. vulgare*, *O. sativa* and several other plant species, but does not increase mutation frequencies in *A. thaliana* and is only marginally mutagenic in humans and animals (Sadiq and Owais 2000). In barley, sodium azide is mutagenic in acidic pH (pH=3) and it primarily induces A/T to G/C transitions. The frequency of chromosome breakage caused by sodium azide is relatively low (Maluszynski et al. 2003). Depending on the species, various concentrations of sodium azide and treatment times are used (for a review, see Gruszka et al. 2011). Apart from a combined treatment with MNU, sodium azide has also been applied in a TILLING experiment in barley, in which three different concentrations (1 mM, 5 mM and 10 mM) of sodium azide were used. Samples treated with these concentrations showed germination rates at similar levels of 85%, 82.5% and 81.3%, respectively. Given the mutation density and acceptable lethal effect, a 10 mM concentration of sodium azide was considered to be optimal (Talamè et al. 2008). The mutation frequency obtained in this experiment was 1 mutation/374 kb and was about three times higher than in the experiment in which EMS was applied as the mutagen (1 mutation/1 Mb) (Talamè et al. 2008 and Caldwell et al. 2004, respectively).

Physical mutagens, gamma-ray radiation and fast neutrons are also applied in functional genomics in plants. In order to create a TILLING population, seeds of *japonica* rice were irradiated with 500 Gy of gamma rays. The average size of the 25 rice genome regions tested in the experiment was 1.2 kb. The mutation frequency was determined as 1 mutation/6.2 Mb. Four of the mutations identified were single nucleotide substitutions and two were 2-bp and 4-bp deletions. Among point mutations, transversions (C/G to A/T and C/G to G/C) are three times more abundant than transitions (mainly C/G to T/A). Taking into account the results of various experiments using gamma radiation, half of the point mutations induced by this mutagen were deletions, which can cause a frameshift if located in exons. The detection rate of knockout mutations generated by this mutagen was much higher than in a TILLING experiment in which chemical mutagens were applied (Sato et al. 2006).

Deletion TILLING (De-TILLING; see below) utilises fast neutron bombardment to develop mutagenised populations (Li et al. 2001). Fast neutrons are a form of high-energy radiation which induces a broad range of deletions and other chromosomal mutations in plants. Several sources of fast neutrons are potentially available for mutagenesis, including particle accelerators and nuclear research reactors. Fast neutrons produced by nuclear fission are accompanied by gamma radiation; however, its contribution is adjustable. To generate short-range secondary particles within the cell nucleus which mediate DNA strand breakage, the neutron energy should be in the range of 500 keV to 5 MeV. De-TILLING mutants offer advantages for crop improvement, as they possess relatively fewer background mutations (Rogers et al. 2009). Deletional mutagenesis may be the best way to knock out tandemly repeated genes, which are common in plant genomes (Achaz et al. 2001; Li et al. 2001).

The two chemical mutagens that are the most widely used in TILLING experiments in animals are *N*-ethyl-*N*-nitrosourea (ENU) and EMS. The former induces both transitions and transversions (G/C to C/G and A/T to C/G); however, G/C to A/T transitions are in the majority (Maluszynski et al. 2003). ENU induces high frequencies of point mutations and is a more potent mutagen than EMS. ENU, which is an alkylating agent, leads to mispairing and, ultimately, results in base pair substitutions and, sometimes, base pair losses (Guénet 2004). In the cases of rats (*R. norvegicus*) and mice (*Mus musculus*), ENU is supplied at the dose of 50–100 mg/kg (Smits et al. 2004 and Nolan et al. 2000, respectively), whereas in a zebrafish (*D. rerio*)

TILLING experiment, the mutagen was applied at a concentration of 3 mM (Wienholds et al. 2003). In rodents, the average density of ENU-induced mutations was 1 mutation/2.3 Mb (in rats) and 1 mutation/1 Mb in mice (Smits et al. 2004 and Nolan et al. 2000, respectively). The density of ENU-induced mutations in the zebrafish genome was significantly higher and was determined as 1 mutation/235 kb (Wienholds et al. 2003).

The second alkylating agent used in TILLING experiments in animals is EMS. However, this mutagen is predominantly used in *D. melanogaster* and *C. elegans*. During the mutagenesis procedure in TILLING experiments in *D. melanogaster*, the mutagen was used at a concentration of 50 mM, whereas in *C. elegans*, the mutagen concentration was 25 mM. As far as mutation densities are concerned, it was determined as 1 mutation/150 kb in *D. melanogaster* (Winkler et al. 2005), whereas the mutation frequency in *C. elegans* was 1 mutation/293 kb (Gilchrist et al. 2006a).

Bioinformatic tools in the TILLING strategy

The bioinformatic tools are used in the TILLING strategy from the beginning, when the candidate gene is the newly identified homologue and the amplicon is determined, till the end, when the analysis of obtained alleles are performed in terms of their impact on protein function. It is important to state that there is no need to know the full genome of the studied species in order to analyse it with the TILLING strategy. The sequence of the gene of interest can be retrieved for many species from databases, such as GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and then a proper homologue can be identified. In the case of barley, whose genome is not fully sequenced, cloning homologues enables their functional analysis. Candidate genes for analysis, when not available for particular species, as e.g. in barley, can be retrieved from GenBank database of Arabidopsis or rice. Afterwards, in the case of Arabidopsis genes, a BLAST search is performed against the rice genome to identify the rice homologue. In order to amplify the coding sequence of a proper homologue, it is necessary to use mRNA and amino acid sequences as a queries in the barley EST databases. The most popular EST databases are TIGR Plant Transcript Assemblies (http://plantta.jcvi.org/), with 456,410 barley EST records (latest update July, 2007), and Computational Biology and Functional Genomics Laboratory (http://compbio.dfci.harvard.edu), with 502,606 barley EST records (latest update March, 2011). EST sequences with the highest similarity are chosen as a template to design primers in order to amplify them. Assembling of the amplified sequences into a barley coding sequence is achieved by using several tools, e.g. CodonCode Aligner (http://www.codoncode.com/aligner), which is able to align and put together contigs into one consensus sequence. The obtained full barley coding sequence is then aligned with the rice genomic sequence in order to establish putative borders between exons and introns. It can be performed using the BLASTN algorithm or software such as Splign (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). The latter additionally makes a graphic model of the gene (Kapustin et al. 2008). It enables the designing of primers in order to confirm experimentally intron presence or absence. Again, the assembling of contigs into a full genomic sequence can be performed with the use of CodonCode Aligner. For the purpose of obtaining the barley gene model, coding and genomic sequences are aligned with the use of BLASTN or Splign software. Based on the coding sequence, the amino acid sequence is determined via *in silico* translation. The last step is the confirmation of the orthology character of a cloned homologue using GreenPhyl (http://greenphyl.cirad.fr/). GreenPhyl contains gene sequences automatically clustered from 12 complete plants' genomes.

The determination of an amplicon is a crucial step for TILLING analysis. The selection of a suitable amplicon provides a higher probability to identify changes in the DNA sequence with an impact on the protein function during TILLING screenings. It is worthwhile to choose a fragment as much as possible within the coding region. The second condition is to identify the region with the most potential to generate deleterious changes. This can be achieved with software such as CODDLE (Codons Optimized to Discover Deleterious LEsions; http://www.proweb.org/coddle/coddle_help.html). CODDLE performs BLAST alignment in order to identify a conserved region, uses SIFT (Sort Intolerant From Tolerant) and PSSM (Position-Specific Scoring Matrix). In addition to CODDLE analysis, the alignment of genomic and amino acid sequences from closely related species with the use of BLASTN and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), respectively, can be performed.

Each of the identified alleles is then analysed in terms of influence protein. A bioinformatic tool designed for displaying and analysing nucleotide polymorphisms is PARSESNP (Project Aligned Related Sequences and Evaluate SNPs; http://www.proweb.org/parsesnp/parsesnp_help.html; Taylor and Greene 2003). PARSESNP determines the effect of single nucleotide polymorphisms (SNPs) on protein, based on the alignment of related proteins with the use of PSSM and SIFT. Sequence alignments are converted to the Blocks format and then to PSSM. PSSM is aligned to the gene with the use of the Multiple Alignment Search Tool (MAST). It determines mapping of PSSM onto a sequence. Large positive PSSM (>10) means that missense change in the analysed sequence of the DNA can be dramatic for protein function. SIFT allows to predict the severity of change; a SIFT score lower than 0.05 could have the same effect as a PSSM higher than 10. Another feature of PARSESNP is

the ability to predict changes in the restriction enzyme recognition sites.

Before starting to phenotype plants carrying mutations identified during the TILLING screenings, it is possible to predict the putative effect of mutation on the secondary structure of proteins. There are several bioinformatic tools used for this purpose. Commonly used are SAS (Sequence Annotated by Structure; http://www.ebi.ac.uk/thornton-srv/databases/sas/; Milburn et al. 1998) and SOPMA (Self-OPtimized Method for secondary structure prediction with Alignment) http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html; Geourjon and Deléage 1995). Both SAS and SOPMA are based on protein alignment algorithms. For proteins containing transmembrane domains, it is worth analysing whether gene products encoded by identified alleles still have these domains. For hydropathy plotting, there are plenty of different software programs available online, e.g. Kyte Doolittle (http://gcat.davidson.edu/rakarnik/kyte-doolittle; Kyte and Doolittle 1982). Kyte Doolittle calculates the hydropathy plot on the basis of the average hydrophobicity of all amino acids of the studied protein (Fig. 2).

Analysis *in silico* is also performed for these mutations that were identified in the non-coding region in terms of putative alternative splicing events. One of the available tools is ASD (Alternative Splicing Database, http://www.ebi.ac.uk/asd/; Thanaraj et al. 2004; Stamm et al. 2006), which analyses introns with SNPs in order to find an impact on the donor/acceptor site, polypyrimidine tract or branch point. It needs to be underlined that all *in silico* data performed with the use of bioinformatic tools has to be verified experimentally by phenotyping each mutant.

TILLING protocol—cheaper over time

Mismatch-specific and sensitive endonucleases

The first strategy that was described for the TILLING procedure (McCallum et al. 2000) included the EMS treatment of Arabidopsis seeds, DNA isolation and pooling, PCR reaction of the fragment of interest, heteroduplex formation and the identification of heteroduplexes using DHPLC. Since then, TILLING has been used with many different organisms and many modifications to the procedure have been introduced that help to automate the screening of mutations and reduce its cost. Over time, the DHPLC method for detecting mutations in the TILLING approach was, in most cases, replaced by the digestion of heteroduplexes using specific endonucleases followed by polyacrylamide electrophoresis and visualisation in the very sensitive LI-COR gel analyser system (LI-COR Biosciences), which is less expensive and faster than DHPLC (Colbert et al. 2001; Till et al. 2006a). Single-stranded specific endonuclease has been used to cleave heteroduplex DNA at the mismatch site in the TILLING procedure. The most popular mutation detection method for TILLING is a mismatch cleavage assay using the endonuclease CEL I from celery in plants (Gottwald et al. 2009), animals (Wienholds et al. 2003) and humans (Till et al. 2006b). CEL I-like nucleases are also found in other plant species: alfalfa, asparagus and tomato. There is one report of using CEL I extracted from the petioles of *B. rapa* (Sato et al. 2006). There are other single-stranded-specific nucleases, such as: S1 from *Aspergillus oryzae*, P1 form *Penicillium citrinum* and mung bean nuclease from the sprouts of *Vigna radiata*, but they are

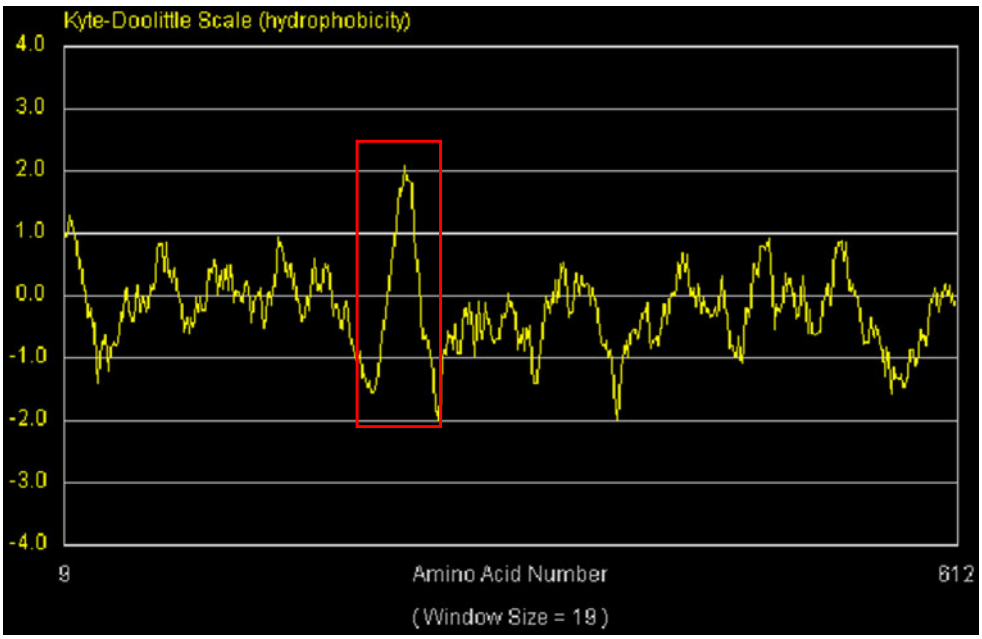

Fig. 2 Example output of analysis using the KD software. A value higher than 1.7 for the window size of 19 indicates the transmembrane domain

mainly active near pH 5.0. The neutral pH optimum incision, which occurred primarily at the phosphodiester bond immediately on the 3′ side of the mismatch and stimulation of activity by a DNA polymerase, are properties that distinguish CEL I from the above-mentioned nucleases (Oleykowski et al. 1998). The successful use of purified nucleases derived from mung bean sprouts (Till et al. 2004b; Kadaru et al. 2006) and *Aspergillus* (S1) (Till et al. 2004b) has also been reported in TILLING. ENDO I from *A. thaliana* is another mismatch-specific endonuclease that belongs to the S1 type. The application of ENDO I has been reported in mutation diagnostics in humans, the fingerprinting of complex populations of viruses, TILLING in *P. sativum* (Triques et al. 2007, 2008) and *S. lycopersicum* (Minoia et al. 2010) and Ecotilling (see below) in *A. thaliana* (Triques et al. 2008).

At the beginning, the most time-consuming step of this method was the cleavage of heteroduplex using a purified enzyme which was, and still is, an expensive reagent. A few years later, it was discovered that crude celery juice extract could also be used for mismatch cleavage and that its endonuclease activity is the same or even higher than in the case of the purified enzyme. The process of preparing it from celery is simple and very cheap, and takes less than two days. The protocol for crude celery juice extract isolation is based on a series of dialyses of the extract directly after juicing the celery. Once isolated, celery juice extract can be used for up to a few years when stored at −80°C (Till et al. 2004b). However, it should be remembered that techniques employing mismatch-specific cleavage enzymes are often limited, as some enzymes do not recognise all mismatch types, have a low sensitivity in detecting one allele in a pool of DNA or lead to a high gel background caused by non-specific cleavage (Triques et al. 2007). To perform TILLING in a cost-effective manner, the purification procedure can also be improved. Sephadex plates can be used as a most effective tool for the purification of PCR products after digestion, but there are also some different methods, e.g. ethanol, which is much cheaper and the efficiency of its action is approximately the same.

Alternatives for LI-COR screening

An alternative method of analysing mutations without using LI-COR screening for which labelled, and, therefore, expensive, primers are required can be the use of non-denaturing polyacrylamide gels with ethidium bromide staining, which was found to have an almost similar sensitivity as traditional LI-COR analysers. This modified TILLING approach was performed in wheat (Uauy et al. 2009). A modified TILLING system using non-labelled primers and fast capillary gel electrophoresis was applied in *O. sativa* (Suzuki et al. 2008). This system has several advantages compared to electrophoresis on a flat glass plate gel: a rapid separation and easier detection of digested fragments on chromatographic images, but a lower sensitivity of detection based on an emission signal from the ethidium bromide intercalation. Also, a mutation scanning method that does not rely on fluorescent primers was implemented by Caldwell et al. (2004). This is based on the double-stranded cleavage of heteroduplex molecules with CEL I nuclease with subsequent fragment detection using a Transgenomic WAVE High Sensitivity denaturing High Performance Liquid Chromatography (WAVEHS dHPLC) system. Another example is the high-throughput capillary electrophoresis system (AdvanceCE™ FS96, Advanced Analytical), which appears to be an investment that can reduce the costs and, most importantly, the time needed to detect mutations. Using this system, the purification step does not have to be performed, which decreases the time needed and the electrophoresis takes about a fifth of the time compared to using LI-COR scanners. Moreover, the PCR reaction is cheaper because the primers used are not labelled, the endonuclease is provided in the reagent kit and the process of loading samples for electrophoresis is automated (C. Weil, personal communication).

Other possible inexpensive alternatives are agarose-based detection systems containing SYBR Green I dye, which was used in *O. sativa* (Sato et al. 2006) or thin agarose gels (<4 mm) with ethidium bromide in *T. aestivum* (Dong et al. 2009). This system may have a lower sensitivity for the detection of mutations in comparison to a system using the LI-COR gel analyser. DEco-TILLING (double stranded Eco-TILLING) is an inexpensive method that involves complete digests of both strands at all mispaired sites in a DNA heteroduplex and was applied to SNP discovery in chum salmon (*Oncorhynchus keta*) using agarose gel electrophoresis with ethidium bromide (Garvin and Gharrett 2007). An alternative approach to mismatch detection was introduced by Cross et al. (2008) for *O. sativa*, EMAIL (Endonucleolytic Mutation Analysis by Internal Labelling), in which amplicon labelling is achieved via the incorporation of fluorescently labelled deoxynucleotides.

Although CEL I-based TILLING is very efficient for detecting mutations, alternative screening platforms have been established, like High Resolution DNA Melting Analysis (HRM) and Conformation Sensitive Capillary Electrophoresis (CSCE). While classical TILLING involves critical and time-consuming steps such as endonuclease digestion reactions and gel electrophoresis runs, when using CSCE or HRM, the only step required is a simple PCR before either capillary electrophoresis or DNA melting curve analysis. HRM depends on the loss of fluorescence from intercalating dyes (LCgreen Plus+™ molecules) bound to double-stranded DNA during thermal denaturation. The release of the dye results in decreased fluores-

cence that is recorded as a melting curve by the LightScanner®. Pools containing mutations form heteroduplexes in the post-PCR fragments mix. These are identified as differential melting temperature curves in comparison to homoduplexes. CSCE depends on the different speeds of the migration of heteroduplexes as compared to homoduplexes. The electrophoresis is performed in ABI 3130×L capillaries filled with a semi-denaturating polymer, thus, allowing the identification of the pools containing a mutation within the targeted fragment. The disadvantage of the CSCE method is the short lengths of the target fragments, between 200 to 500 bp, compared to CEL I LI-COR platforms, where it is possible to screen for fragments up to 1.5 kb in one run. The CSCE and HRM methods were used for mutation screening in tomato plants (Gady et al. 2009). Combining HRM scanning with sequence analysis using Mutation Surveyor was applied in wheat (Dong et al. 2009). Mutation Surveyor is commercially available software for DNA variation analysis that allows automatic mutation detection in sequence traces. HRM analysis was also employed for the detection of mutations in the medaka (*Oryzias latipes*, Japanese killifish) TILLING library (Ishikawa et al. 2010). The same technique (HRM) was used in iTILLING (see below) (Bush and Krysan 2010).

iTILLING

A new approach to the TILLING method that reduces costs and the time necessary to carry out mutation screening was developed for Arabidopsis and it is called iTILLING, individualized TILLING (Bush and Krysan 2010). In the traditional method, the M_2 plants are grown in soil and DNA is isolated from them individually and then pooled in order to perform PCR-based mutation detection (McCallum et al. 2000). With the iTILLING strategy, seeds from M_1 are collected in bulk and cataloguing of the plants is not necessary. The M_2 plants are grown one or two per well on 96-well spin plates on agar plugs and the tissue for DNA isolation is harvested directly from the plates using the Ice-Cap method (Krysan 2004; Clark and Krysan 2007). The name Ice-Cap indicates that ice is used to capture the tissue samples. The roots of seedlings grow through the agar towards a second 96-well plate that is filled with water and, after approximately 3 weeks, reach the bottom of the second plate. The lower plate with the root fragments inside is then frozen and separated from seedlings. This plate serves as a platform for DNA isolation from the root tissue. The seedlings of plants remain intact. Isolated DNA is directly used as a template for PCR reactions and the mutation screening procedure can be performed. In the case of iTILLING, a high-resolution melt curve analysis of amplified fragments is performed in order to reveal mutations without using enzymatic cleavage and gel electrophoresis. Since there are two plants grown in each well, the isolated DNA is already pooled 2-fold; therefore, the identification of mutations in both heterozygous and homozygous states is possible. When seeds are sown one per well, only heterozygous mutations can be detected. After the mutation is discovered, the corresponding seedling(s) is transferred from the 96-well plate to the soil and grown in order to characterise its phenotype and to produce further generations (Bush and Krysan 2010). The same strategy of pooling tissue samples instead of nucleic acid from individual plants in the 96-well plate followed by DNA isolation from the arrayed samples was recently reported in tomato plants as NEATTILL, a simplified procedure for nucleic acid extraction from arrayed tissue for TILLING (Sreelakshmi et al. 2010). The iTILLING strategy is supplemental to the traditional TILLING approach. Although it reduces the investment required, it can only be performed for a small number of genes because the screening population used is of a short-lived nature and only plants with mutations that can be identified in a relatively short time can be grown to reach maturity and yield seeds for storage. The iTILLING strategy has already been successfully applied to many different species in a number of laboratories. Because the cost of this method has been reduced over time, iTILLING could well become the most common strategy of reverse genetics.

Variant discovery in plants using next-generation sequencing technologies

The advances in DNA sequencing technologies have been used in two ways in the TILLING strategy. Firstly, the growing number of whole-genome sequencing projects (for a review, see Mochida and Shinozaki 2010) in plants for both crop and model species has led to an increase in the possible choices of target genes in TILLING. Secondly, new strategies for sequencing, so called next-generation sequencing methods (NGS), which has been applied to a limited number of sequenced individuals using Sanger's method, can be used for direct mutation determination without any pre-screening. The advent of NGS platforms has dramatically increased the speed at which a DNA sequence can be acquired and has also reduced the cost of sequencing by more than two orders of magnitude (Deschamps and Campbell 2010). The NGS platforms, which are available today, include the 454 Genome Sequencer FLX and its smaller version, the GS Junior System, both of which are pyrosequencing-based instruments (Roche Applied Science; http://454.com/products/index.asp), the Solexa 1 G Genome Analyzer (Illumina; http://www.illumina.com), the SOLiD instrument (Applied Biosystems; https://products.appliedbiosystems.com) and

the HeliScope Single Molecule Sequencer (Helicos; http://www.helicosbio.com).

Application of NGS in TILLING

Although there are not many examples of using NGS in the TILLING strategy for mutation detection to date, the situation may be much different in the future because the potential of those methods is great. The cost of the required equipment and reagents are still the drawbacks that limit their application. The number of nucleotides which can be sequenced in one run is large, but the number of identification tags ("sample bar coding") is limited. The technical requirements are also quite high. The analysis of the results of sequencing using NGS methods is also not very simple because it is necessary to distinguish between the false-positive and real SNPs. Rigola et al. (2009) used the 454 Roche technology for mutation detection in a tomato population obtained after EMS treatment and identified two mutations in the *eIF4E* gene based on the screening of more than 3,000 M_2 families in a single GS FLX sequencing run. There is also information on the website on the TILLING service, UC Davis TILLING Core. They have converted from LI-COR-CELI assays for the detection of mismatches to the NGS of pooled genes using the Illumina GAII platform (http://tilling.ucdavis.edu/index.php/How_to_Get_TILLING). The same platform was used by the teams of L. Comai (University of California, Davis); J. Dubcovsky (University of California at Davis); S. Henikoff (Fred Hutchinson Cancer Research Center, Seattle); R. Tran and D. Lin (University of California, Davis), working under the project DBI-0822383 "Efficient identification of induced mutations in crop species by ultra-high-throughput DNA sequencing" of the National Science Foundation, USA, which has led to the application of this technology in TILLING as well.

Modifications of the TILLING strategy

Ecotilling—discovery of SNPs in natural populations

One of the first modifications of the TILLING strategy, called Ecotilling, was proposed by Comai et al. (2004). In this modification, mutation detection technology was used to discover polymorphisms in a natural population of *A. thaliana*. Instead of using pools of DNA from mutagenised plants as templates, the DNA of *A. thaliana* ecotypes, each mixed with a reference Columbia ecotype DNA, was used. The authors discovered 55 haplotypes in five genes in more than 150 individuals, ranging from sequences differing by a SNP to those representing complex haplotypes. Ecotilling enables the rapid detection of variations in many individuals and is cost-effective because only one individual for each haplotype needs to be sequenced. The technology is applicable to any organism, including those that are heterozygous or polyploid. The next reports for a cultivated species appeared a short time later, and it was found out that this method could have practical applications in plant breeding, e.g. in the searching for resistance to new viruses or to create genetic diversity, which is important from an agronomic point of view. The wild relatives of cultivars, which have limited genetic diversity, could also be explored using this method and natural alleles could be detected. In the future, the genetic resources discovered by Ecotilling can be exploited in new cultivars. To date, many important EcoTILLed genes have been screened in natural populations of different species: *FAE1* (*fatty acid elongase 1*), which is involved in the control of erucic acid synthesis in *Brassica* species (Wang et al. 2010); *Alk*, which encodes soluble starch synthase IIa in *O. sativa* (Kadaru et al. 2006); *Ara d 2.01* (conglutin gene), the orthologue of *Ara h 2.01*, which codes the seed storage protein in *Arachis duranensis*, which is a very potent allergen for humans (Ramos et al. 2009); and *Pina-D1* (*puroindoline a*) and *Pinb-D1* (*puroindoline b*), which mainly condition kernel hardness through allelic variations in these genes in *T. aestivum* (Wang et al. 2008b). Another important target for agriculture could be a resistance to herbicides. Several classes of herbicides are known to inhibit the *ALS* (*acetolactate synthase*) gene. These highly selective *ALS*-inhibiting herbicides are very valuable for the weed management for a wide range of crops worldwide. Ecotilling was used for the detection of single nucleotide mutations in the *ALS* genes of sulfonylurea (SU)-resistant (R) *Monochoria vaginalis* (Pontederiaceae), a paddy weed in Japan. Genomic DNA of an SU-R plant (target DNA) was mixed with the DNA of an SU-susceptible (S) plant (reference DNA). Ecotilling detected two nucleotide mutations in the *ALS* gene of SU-R *M. vaginalis*; this type of mutation has been reported to result in insensitivity to SUs in many weed biotypes (Wang et al. 2007).

Another valuable application of Ecotilling is searching for new virus-resistant alleles because natural populations are rich genetic resources and could be used to reach this goal. Translation initiation factors of the 4E and 4G protein families mediate resistance to several RNA plant viruses in natural crop diversity (Nieto et al. 2007). Nucleotide changes in the genes *eIF4E* and *eIF(iso)4E* of translation initiation factors have been detected in *Capsicum annuum* (Ibiza et al. 2010). Moreover, the utility of these new allelic variants have been demonstrated by testing them for PVY (Potato virus Y) resistance. Five new resistance alleles of the *eIF4E* gene have been detected. The same gene *eIF4E*, which controls resistance to MNSV (Melon necrotic spot virus), was screened in *Cucumis* species (Nieto et al. 2007). One new allele from *Cucumis zeyheri*, a wild relative of

melons, has been characterised and it may be responsive for resistance to MNSV. Ecotilling was also used to identify allelic variation within the powdery mildew resistance genes *mlo* and *Mla* in *H. vulgare* (Mejlhede et al. 2006). This method not only confirmed the appearance of different alleles, but it also found that these differences can be used as a powerful genetic marker. Ecotilling offers the possibility of combining different *mlo* alleles with different *Mla* alleles in order to obtain cultivars with a more durable resistance. Ecotilling has also been used in *Solanum tuberosum* (Elias et al. 2009) and *Musa* species diploid and polyploid accessions (Till et al. 2010).

Ecotilling could also be used in animal species as well as in plant species. Rare human nucleotide polymorphisms were discovered using this method (Till et al. 2006b). An ongoing problem in the application of Ecotilling for large mutation screening projects in humans is the lack of bioinformatics support for a quick and comprehensive evaluation of each newly found SNP. Coassin et al. (2008) proposed a solution to deal with the results and interpreting the possible functions of new variants by applying freely available software tools.

Ibiza et al. (2010) reported a modification of Ecotilling in which cDNA instead of genomic DNA was used. This approach avoids intron sequences and reduces the difficulty of amplifying target genes from different species. However, non-coding regions provide more variation and may, therefore, be more useful for mapping and genotyping (Gilchrist et al. 2006b).

Ecotilling in ecology

The ecotilling strategy could be used in ecology for studying population structures and evolutionary relationships. Wang et al. (2010) collected 116 accessions of modern cultivars of *B. napus*, *B. rapa* and *B. oleracea*. Variations in two paralogues, *B. napus FAE1* (*fatty acid elongase 1*), among these cultivars were detected using Ecotilling. A phylogenetic tree of *FAE1*s indicated that the divergence between the A and C genome occurred later than the one between *Arabidopsis* and *Brassica* species; 18 SNPs were found in the coding region of *FAE1* and may be used as genome-specific markers to distinguish the A and C genomes. Ecotilling was also used as a tool to examine DNA variation in natural populations of *Populus trichocarpa*. Nine different genes among individuals from 41 different populations were screened. A clear geographic pattern of the population structure of *P. trichocarpa* was established (Gilchrist et al. 2006b).

Other modifications of the TILLING strategy

An interesting modification of the TILLING method, called De-TILLING (Deletion TILLING), was reported by Rogers et al. (2009). This method expands the spectrum of available reverse genetics molecular tools for the functional characterisation of genes. In contrast to TILLING, which provides an efficient method for the identification of mainly single base pair mutations from which only approximately 5% will be null mutations, De-TILLING overcomes this shortage and exclusively detects knockout mutations. The first report in which agarose gel electrophoresis was used for deletion detection in a fast neutron treated population of Arabidopsis used the Delete-a-gene® approach (Li et al. 2002). This approach is based on suppressing the amplification from wild-type sequences in order to identify specific deletion alleles in a large population of plants. Twenty-five loci were screened for deletion mutations in a population containing a total of 51,840 lines. Deletion mutants were identified at a rate of 84%, which indicates that Delete-a-gene® can be used very effectively to knockout target genes. In crop plants, deleting unwanted genes can also be used to create improved cultivars.

Physical mutagenesis with fast neutrons has also been applied in a large population of 156,000 *M. truncatula* plants for De-TILLING. This population was structured and 13 super-pools were created, each representing 12,000 M_2 plants. A large population size in De-TILLING is compensated by a high level of pooling. A sensitive PCR-based detection was used in order to discover null mutation in targeted genes. The screening of 14 genes in this population using five targets per gene led to the identification of mutants at a rate of 29%. The screening was carried out using an average target size of 2.3 kb and the identified deletions removed from 18% to 68% of the target regions. Another example of using this platform for the detection of deletion in homoeologous genes in wheat was reported by Fitzgerald et al. (2010). Other physical mutagens like heavy ion irradiation (HII) have also been employed for the creation of a population. The TaqMan SNP detection method was used to identify homoeologous deletion mutants. This method relies on uniquely fluoro-labelled homoeologue-specific TaqMan SNP detection probes to identify the presence or absence of homoeologous copies of the gene of interest. This solution overcomes the technical difficulties in the application of TILLING to polyploidy crop species due to the independent amplification of homoeologous gene copies, which prevents the formation of heteroduplexes between these closely related sequences.

Although LI-COR-based techniques are commonly used for mutation screening, MALDI-TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry)-based assays have been reported for mutation detection in an oat TILLING population. In MALDI-TOF, samples are ionised and then relocated to the mass analyser, where they are separated according to their mass-to-charge ratio. The ions are then detected and analysed using specially developed software. However, there are few drawbacks with the current

Table 5 Examples of using the TILLING strategy in functional genomics and plant breeding

Species	No. of M_2 screened	Target trait	Target gene	No. of identified mutations	Reference
Avena sativa	350	Increased digestibility	*AsPAL1* (*phenylalanine ammonia-lyase*)	6	Chawade et al. 2010
	426	Improved food quality	*AsCslF6* (*cellulose synthase-like*)	10	
Brassica napus	1,344	Oil quality	*FAE1* (*fatty acid elongase 1*)	19	Wang et al. 2008a, b
Brassica oleracea	960	Glycolysis	*PgiC* (*glucose-6-phosphate isomerase*)	4	Himelblau et al. 2009
Brassica rapa	3,072	DNA methylation	*MET1* (*methyltransferase1*)	183	Stephenson et al. 2010
Cucumis melo	3,306	Enhanced shelf life	*CmACO1* (*ACC oxidase 1*)	7	Dahmani-Mardas et al. 2010
Hordeum vulgare	7,389	Row-type morphology	*HvHox1* (*homeodomain-leucine zipper I-class homeobox protein*)	31	Gottwald et al. 2009
	1,920	Immunity to fungus	*Mlo9* (*mildew resistance locus 9*)	4	
	3,148	Virus resistance	*eIF4E* (*eukaryotic translation initiation factor 4E*)	5	Talamè et al. 2008
	3,148	Immunity to fungus	*Rpg1* (*barley stem rust resistance protein gene 1*)	4	
Lotus japonicus	4,904	Nodule development	*NIN* (*nodule inception*)	4	Perry et al. 2009
Oryza sativa	767	Leaf emergence	*PLA1* (*plastochron 1*)	14	Suzuki et al. 2008
Sorghum bicolor	768	Forage digestibility	*COMT* (*caffeic acid O-methyltransferase*)	2	Xin et al. 2008
Solanum lycopersicum	4,759	Virus resistance	*eIF4E1* (*eukaryotic translation initiation factor 4E*)	7	Piron et al. 2010
	5,169	Enhanced shelf life	*RIN* (*ripening inhibitor*)	12	Minoia et al. 2010
Solanum tuberosum	864	Starch quality	*GBSSI* = the Waxy protein (*granule-bound starch synthase I*)	19	Muth et al. 2008
Triticum aestivum	630	Grain hardness	*Pina*, *Pinb* (*puroindoline a, b*)	18	Feiz et al. 2009
	981	Starch quality	*Sgp-1* (*starch granule protein 1*)	13	Sestili et al. 2010

MALDI methods, e.g. its reliance on specialised equipment and software for mutation identification without automation. MALDI screening is still a low-throughput method with high costs of the chemicals needed for large-scale screening programmes (Chawade et al. 2010).

New alleles identified using TILLING platforms and their importance in studying gene function and crop improvement

Theoretically, the TILLING strategy has led to the creation and identification of a series of new alleles for any gene of interest in any plant species. However, in order to achieve this goal, certain conditions need to be fulfilled. The most important is to perform proper mutagenic treatment, which will result in a high density of mutations, as well as in a good survival rate. These two factors need to be in balance. Secondly, the M_2 population needs to be large enough to make it possible to find new alleles of any of the studied genes. The creation of a mutant population for vegetatively propagated species or with a long generation time is much more challenging. For such species, Ecotilling may be the best solution, which was shown in research conducted on *Musa* (Till et al. 2010). Finally, it should be possible to detect any allelic series causing different changes in a coded protein for any gene and to use it to study gene function. The newly identified alleles could also be used as valuable resources in crop improvement. To date, there have been many successful examples of both approaches (Table 5). Perry et al. (2009) identified a large allelic series for 12 genes known to be essential for nodule development in *L. japonicus*. A total population of 4,904 M_2 plants was screened and 97 mutant alleles were detected. All possible types of mutation were identified: silent alleles that caused no change in amino acid, changes in splice sites and missense to nonsense types. This unique data set, which combines genotypic and phenotypic information, is an excellent tool for structure–function studies. Among the mutants identified, 19 alleles did not have an effect on gene function and 78 influenced the phenotype, including lines where nodulation deficiency was observed. Another investigated trait was spike morphology (Gottwald et al. 2009). A TILLING population in barley was created using the two-rowed malting cultivar 'Barke'. Thirty-one mutations were identified by screening a 1,270-bp fragment of the homeodomain leucine zipper (HD-ZIP) gene *HvHox1* in 7,348 M_2 lines. Three of the newly identified mutants exhibited either a six-rowed or an *intermedium*-spike phenotype, and these mutations constituted a direct link between the gene and the phenotype. Reverse genetic screening of mutagenised populations could be also used as a molecular tool for crop improvement. Enhanced shelf life was the goal for research in *Cucumis melo* (Dahmani-Mardas et al. 2010). TILLING screening in melons were performed for 4,032 M_2 plants and 11 genes related to fruit quality were chosen. In total, they identified and confirmed by sequencing 134 induced mutations in an 18.3-kb total length of tilled amplicons. A detailed investigation was performed for *CmACO1*–ACC oxidase 1, the enzyme that catalyses the last step of ethylene biosynthesis and is connected with the shelf life of fruit. One mutation out of the seven detected in this gene, G194D, occurred in a highly conserved amino acid position and an assumption was made using crystallographic analysis that it affects the enzymatic activity. A phenotypic analysis confirmed this assumption that the mutant showed a significant delay in ripening and yellowing, with improved shelf life. Among the broad range of genes that encode traits that are of great interest to breeders, key genes in the lignin and β-glucan biosynthetic pathways were chosen in *A. sativa* (Chawade et al. 2010). Currently, oats are mainly used as feed for animals. A lower lignin content will increase grain digestibility, which, in turn, will increase the feeding value of the crop. However, oats rich in β-glucan have properties that are beneficial to the health of humans, such as those that improve digestion and help to lower cholesterol. Therefore, β-glucans are becoming very important food ingredients; hence, there is much more interest in oats as a food for humans. In an oat cv. 'Belinda' TILLING population, six different mutations in the phenylalanine ammonia-lyase (*AsPAL1*), which is a key gene in lignin biosynthesis, and ten different mutations in the cellulose synthase-like (*AsCslF6*) β-glucan biosynthesis gene were detected. Among them, 50% of the newly identified alleles in each analysed gene led to an amino acid change. Only a bioinformatic analysis of the identified mutation was carried out. Based on the SIFT and PSSM scores, it is more likely that mutations detected in the *AsPAL1* gene rather than in the *AsCslF6* gene may cause any phenotypic changes. However, this assumption needs to be proved by phenotypic tests. The alleles generated by the TILLING method might be used as new resources in the improvement of oats.

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