TILLING AND FOX-HUNTING: NEW METHODS FOR FUNCTIONAL ANALYSIS OF GENES

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Summary: Theoretical and experimental bases of TILLING and FOX-hunting, new tools for precise identification of genes in functional studies are reviewed. TILLING (Targeting Induced Local Lesions IN Genomes) is a technique combining chemical mutagenesis with its sensitive and precise detection. The method involves PCR amplification of DNA samples pooled following extraction from a collection of chemically-treated organisms and a subsequent screening for mutations using *Cel*1 endonuclease, detecting mismatches in heteroduplexes [52]. FOX-hunting (Full-length cDNA Over-eXpressing gene hunting system) is a new method of plant gene overexpression, which enables a quick gene isolation and sequencing, paralelly with functional studies.

Key words: TILLING, FOX-hunting, functional analysis of genes, mutagenesis

INTRODUCTION

The beginning of the twenty-first century has been called post-genomic era as more than 180 complete genome sequences started from the sequence of the phage ΦX174 in 1977 (5,368 bp), *Haemophilus influenzae* in 1995, the fruit fly and *Arabidopsis* in year 2000, a man in 2001, rice in 2002, and in 2007 the first complete genome, 6 billion nucleotides, of one man [22] have been known. Knowledge of genomes sequences is not eqiuvalent with the knowledge about gene number and function. For example, the rice genome which is the smallest among cereals at 430 Mbp, and thus assumed to be a model for plant genome sutdies, was *in silico* predicted to encode approximately 50 000 potential genes, mostly of unknown function.

For more than a decade, comprehensive data describing changes in gene expression profiles (mainly quantitative) have been collected. In 1996, the biotech

company, Affymetrix, produced the first commercial DNA microarray. Identification of differentially expressed mRNAs using microarray technology generates enormous amounts of data. For example, a microarray experiment using cDNA derived from drought tolerant and sensitive rice varieties identified approximately 16 000 genes affected by drought stress, two thirds of which have no known function [5]. Similarly SAGE (**S**erial **A**nalysis of **G**ene **E**xpression) developed in the mid-nineties, and MPSSE (**M**assively **P**arallel **S**ignature **S**equencing) developed in the year 2000, which allow the mass sequencing of cDNAs fragments, generate large data sets [21, 24]. To sort and organize the large amount of experimental data which is still growing, there is a need for rapid expansion of knowledge about gene function, as well as new tools for construction and handling of multi-parameter databases [25, 33 and 34].

For many years functional analyses of genes were carried out according to principles of classical genetics, from the phenotype to the gene (Top-Down/Forward Genetics). The development of molecular biology techniques has allowed the opposite way: from a mutation to a phenotype (Bottom-up/Reverse Genetics) (Table 1). Functional analysis of genes sped up with the development in the U.S. of TILLING platform (**T**argeting **I**nduced **L**ocal **L**esions **IN G**enomes) in the mid 90's, which allows massive and rapid identification of point mutations basing on DNA amplification and specific digestion [36]. The rate of accumulated functional data has also increased due to the development of the FOX-hunting method (**F**ull-length cDNA **O**ver-**E**xpressing gene hunting system) in Japan. This method, patented in 2001 and made available to a broader range of researchers in 2008, allows for quick gene isolation and functional identification under conditions of gene over expression [17]. The dynamic developments of gene functional analysis has therefore provided the inspiration for overview of a conceptual framework of TILLING and FOX-hunting techniques on the background of the classical forward genetic screen.

FUNCTIONAL ANALYSIS OF GENES ACCORDING TO THE PRINCIPLES OF CLASSICAL GENETICS

In accordance to the principles of a classical forward genetics screen, functional analysis of genes involves identifying a gene which encodes a specific protein. Positional cloning of the gene is the example of such an approach. This method requires finding the molecular markers closely linked to the trait of interest. Depending on the nature of the trait, different methods of mapping are applied: qualitative or quantitative (QTL or associative, based on an analysis of Linkage Disequilibrium (LD)). For plants, mapping is accomplished based on segregating populations: BC_2 or F_2 (Back Crossed or regularly fertilized 2^{nd} generation of offspring), doubled haploid lines (DH), recombinant lines (RILs) as

well as near-isogenic lines (NILS), aneuploids or/and substitution lines [15]. Molecular markers which strongly co-segregate with the trait, are placed on the physical maps generated from contigs of clones selected from genomic libraries. After the region of DNA located between the flanking markers is sequenced, selected DNA fragments are isolated and cloned into transformation vectors. Changes in the phenotype of mutant plant gained by transformation, which are consistent with the expected result, confirm the biological function of the gene. Lack of expected phenotypic changes however is uninformative as it may be the result of gene silencing in transformants [27]. An example of such studies might be a search for a gene *Pi-ta2* encoding resistance to *Pyricularia grisea* in rice [31]. In wheat genome several genes has been identified by positional cloning. While majority of these are genes of resistance to stresses and response to vernalization, on chromosome 5AL a regulatory locus involved in the domestication has been identified. This locus encodes genes of high gluten content in grain, as well as locus of homologous chromosomes conjugation during meiosis (Table 2).

A more efficient approach to functional gene analysis is to create, identify and then analyze mutants. Mutagenesis was introduced into breeding in the early thirties, when the possibility of mutant induction by X-ray was discovered. The development of molecular biology techniques allowed for use of this approach to find genes and to understand their functions without knowledge of phenotype and /or protein products.

TABLE 1. Comparison of methods of gene functional analysis: Top-down vs. Bottom-up approaches [30, modified]

Functional analysis of genes	
Top-down (classical)	*Bottom-up (molecular)*
PROTEIN / PHENOTYPE	PROTEIN / PHENOTYPE
⇩ Mapping: QTL / associative	⇧ Protein overproduction, isolation and analysis
GENE / GENES	GENE / GENES
⇩ Transformation	⇧ Gene identification
MUTANTS	MUTANTS
⇩ Phenotype identyfication	⇧ Phenotype identification
COMPLEMENTARY TEST	MUTAGENESIS

TABLE 2. Wheat genes identified from positional cloning projects [12, modified]

Gen/QTL	Chromosome localization	Trait	University/ Group lider
Lr1	5DL	Leaf rust resistance	University of Zurich/ B. Keller
Lr10	1AS	Leaf rust resistance	University of Zurich/ B. Keller
Lr21	1DS	Leaf rust resistance	Kansas State University/ B. S. Gill
Qfhs.Ndsu-3bs	3BS	Fusarium head blight resistance	Kansas State University/ B. S. Gill
Pm3b	1AS	Powdery mildew resistance	University of Zurich/ B. Keller
Yr5	2BL	Resistance to stripe rust	USDA-ARS, Wheat Genetics/ K. G Campbell
Sr2	3BS	Stem rust resistance	CSIRO Plant Industry, Australia/ E. S. Lagudah
Tsn1	5BL	Host-selective toxin *Ptr ToxA*	North Dakota State University/ J. D. Faris
B	7BL	Boron tolerance	University of Adelaide/ P. Langridge
Fr2	5BL	Frost resistance	University of California, Davis/ J. Dubcovsky
VRN1	5A	Vernalization response	University of California, Davis/ J. Dubcovsky
VRN2	5A	Vernalization response	University of California, Davis/ J. Dubcovsky
VRN-B3	7BS	Vernalization response	University of California, Davis/ J. Dubcovsky
EPS-1	1AL	Flowering time	University of California, Davis/ J. Dubcovsky
Q	5AL	Free threshing character	Kansas State University/ B. S. Gill
GPC-B1	6BS	High grain protein content	University of California, Davis/ J. Dubcovsky
Ph1	1A/5B	Chromosome pairing locus	John Innes Centre, Colney, Norwich/ G. Moore

FUNCTIONAL ANALYSIS OF GENES ACCORDING TO THE PRINCIPLES OF MOLECULAR GENETICS

Methods for induction of mutants

Physical mutagenesis

Physical mutagenic agents include mostly different types of radiation: gamma (source: radioactive isotopes), X (sources: X-ray tubes, synchrotrons), ultraviolet and electrons with high and low kinetic energy (source: accelerators) [3]. Radiation energy alters the nature of chemical and short-distance physical bonds existing between atoms in bio-polymers, leading to the destruction of proper ones by the formation of invalid, which finally results in disturbances of DNA metabolism and mutation formation. The phenomenon of X-ray-induced mutagenesis was first described for the barley genome during interwar period. Comprehensive programs for diversity generation by physical mutagenesis in collections of crop plants were undertaken in the '60s. The cooperation of FAO (**F**ood & **A**griculture **O**rganization) and IAEA (**I**nternational **A**tomic **E**nergy **A**gency) in the peaceful uses of atomic energy has resulted in many breeding programs worldwide. Since then, 2.570 mutants were registered throughout the world, including the 1020 mutants of major cereal crops: 439 mutants of rice, 305 mutants of barley, 204 mutants of wheat and 71 mutants of maize [26]. Most of those mutations were induced by γ-rays (30%) and to a lesser extent by the X-rays (3%). Two thirds of these mutants were generated in China. Notably, for the spectacular achievements of physical mutagenesis belongs barley variety well yielding on the attitude above 5 000 m above sea level in Andes in Peru. To natural phenomenon generated by mutagenesis belongs also the variety of rice that can grow in water of high salinity, in the Mekong Delta in southern Vietnam [38]. In Poland, significant achievement of γ-ray mutagenesis was self-ending mutant of horse bean (*V. faba* var. *equina*) [1].

Chemical mutagenesis

Chemical mutagenesis (mostly point mutation) occurs under the influence of conformational changes in one pair of nucleotides in the complementary chains of DNA. Chemicals that cause conformational changes include analogues of nucleotide bases (5-bromouracil or 2-aminopurine); hydroxyl agents (hydroxylamine); alkyl reagents (ethyl methanesulfonate or dimethylnitrosamine); deamination reagents (nitrous acid or sodium sulfite). Intercalation of aromatic compounds molecules, such as proflavine or ethidium bromide, into the DNA helix may also lead to disruptions in DNA replication, repair, or recombination.

The likelihood of generating a dominant point mutation in the cereals is less than half permil [18]. Transparent example of the use of chemical mutagenesis are stidies of Finkelstein group, which used *A. thaliana* mutants insensitive to abscisic

acid and explained many aspects of signal transduction on the hormone-dependent path [9]. An important achievement of Polish breeders was obtaining of winter oilseed rape forms characterized by increased content of oleic acid and reduced content of linolenic acid in the seeds of mutagenesed plant in comparison to the double-improved varieties [32].

TABLE 3. Existing and proposed projects of gene functional analysis in grass species based on chemical mutagenesis and TILLING analytical system [37, modified]

Cereal Crop	Project	Mutagen	Web- page address
BARLEY			
cv. Optic	DIStilling (SCRI)	EMS	http://germinate.scri.sari.ac.uk/barley/mutants/
cv. Barke	GABI-TILL	EMS	www.gabi-till.de/project/ipk/barley.html
cv. Morex	TILLMore	EMS	www.distagenomics.unibo.it/TILLMore/
cv. Lux	Risø National Labs, KVL Denmark	EMS	www.pgrc.ipk-gatersleben.de/barleynet
MAIZE	Maize TILLING Project, Purdue	EMS	http://genome.purdue.edu/maizetilling/
OAT	CropTailor AB	EMS	www.croptailor.com/Englesk/engindex.htm
RICE	RiceTILL (UC Davis)	EMS or MNU + NaN_3	www.tilling.ucdavis.edu/index.php/Rice_Tilling
(*ssp*. *japonica*)	Mishima	MNU	
WHEAT			
T. aestivum	Arcadia Biosciences	EMS	
T. monococcum	Rothamstead Research (RRes)	EMS	http://www.rothamsted.bbsrc.ac.uk/ppi/staff/hcj.html
T. durum	OPTIWHEAT	EMS	www.rothamsted.ac.uk/cpi/optiwheat/indexcontent.html
SORGO	USDA, Lubbock, TX	EMS	
SWITCHGRASS	Purdue TILLING Project	EMS	http://genome.purdue.edu/
BRACHYPODIUM	Risø National Labs, KVL Denmark	NaN_3	www.risoe.dk/rispubl/BIO/biopdf/ris-r-510.pdf.

For over half a century, chemical mutagenesis has been an important method for generating mutants, and in the last decade, due to the development of TILLING platform, new comprehensive research projects have been established (Table 3).

Insertional mutagenesis

Insertional mutants are obtained by transformation of wild plants with naturally occurring retrotransposons or T-DNAs [20, 35]. Mutants generated from these methods typically arise from gene silencing (called: knock out or loss-of-function) due to insertion into the coding sequence [40]. Naturally occurring insertional mutants were first analyzed in maize due to the presence of active transposon elements [23]. This discovery led Barbara McClintock to be awarded a Nobel Prize (1948). Cloning of *Ac* and *Ds* transposons of maize [7] enabled the transformation of species whose genomes have not active transposon system. The first gene isolated using transposon tagging, was the tomato Cf-9 gene required for resistance to the fungus *Cladosporium fulvum* [16]. Such a strategy of functional gene analysis dominated for nearly two decades in laboratories worldwide, so it's not surprising that the number of generated, in this way, mutant plant is more than 290 000 [11]. Factors limiting the use of insertional mutagenesis include the inability to analyze the genes when multiple copies are inserted and also the inability to study genes expressed in early stages of plant development due to embryo lethality. In addition, these mutants, in contrast to the T-DNA mutants, are unstable, which is a direct result of the properties of transposons. T-DNA is integrated into the genome of a plant with an average of 1.5 copies in the genome of *Arabidopsis* or rice [8]. Table 4 summarizes the available research collections of insertion mutants in rice.

In general, transformations with either transposons or T-DNA lead mainly to recessive mutant. Therefore, selecting a suitable mutant for further study requires a number of crosses and analysis of the phenotypes of many offsprings; in case of *Cf-9* gene approximately 160 000 mutants of tomato individuals were studied [16].

Increased transformation efficiency coupled with simplifications in systems of plant regeneration and mutant identification resulted in development of the FOX-hunting system. This system does not require *in vitro* cultures, as embryos in young inflorescences, dipped in a solution of *Agrobacterium tumefaciens*, are transformed with a T-DNA binary vector [4]. T-DNA binary vectors used in these experiments carry a full-length cDNA of the gene of studied organism [14]. This promotes the generation of mutants characterized by ectopic gene expression which is also referred to as the gain-of-function (Fig. 1).

In recent years, RNAi technology has become a powerful tool for functional analysis of genes, however, as it is based on post-translation gene silencing [27, 29] it is not the object of the present article.

TABLE 4. Rice mutant resources [19, modified]

Institution	Genotype	MMutagen	Mutated loci			Web site	Leader/e-mail address
			total	classified	available		
POSTECH *South Korea*	Dongjin, Hwayoung	T-DNA ET/AT Tos17	150 000 400 000	84 680	58 943	RISD http://an6.postech.ac.	G. An genean@postech.ac. kr/pfg
CIRAD- INRAIRD-CNRS, Genoplante *France*	Nipponbare	T-DNA ET Tos17	45 000 100 000	14 137 13 745	17 414 11 488 (03.`09)	http://urgi.versailles.inra.f r/OryzaTagLine	E. Guiderdoni guiderdoni@cirad.fr
IPMB, Academia Sinica *Taiwan*	Tainung 67	T-DNA AT	30 000	18 382	31 000	TRIM http://trim.sinica.edu.tw	Y.C. Hsing bohsing@gate.sinica. ed.tw
Huazhong Agricultural University *China*	Zhonghua 11 Zhonghua 15 Nipponbare	T-DNA ET	113 262 14 197 1 101	16 158	26 000 (12.`08)	RMD http://rmd.ncpgr.cn	Q. Zhang qifazh@mail.hzau.ed u.cn
SIPP *China*	Zhonghua 11	T-DNA ET	97 500	8 840	8 840 11 000	http://ship.plantsignal.cn/ home.do	F. Fu ship@sibs.ac.cn
Zhejiang University *China*	Nipponbare Zhonghua 11	T-DNA		1 009	1 009	http://www.pi.csiro. au/fgrttpub	P. Wu clspwu@zju.edu.cn

TABLE 4. Rice mutant resources [19, modified]

Institution	Genotype	Mutagen	Mutated loci			Web site	Leader/e-mail address
			total	classified	available		
NIAS *Japan*	Nipponbare	Tos17	500 000	34 844	34 844	http://tos.nias.affrc.go.jp	H. Hirochika hirohiko@nias.affrc.go.jp
UC Davis *USA*	Nipponbare	Ac-Ds GT Spm/dSpm	20 000	Ds 4 735 dSpm 9 469	4 630 9 036	http://www-plb.ucdavis.edu/ Labs/sundar	V. Sundaresan sundar@ucdavis.edu
Gyeongsang National University; *South Korea*	Dongjin Byeo	Ac-Ds GT	30 000	4 820	4 820	KRDD http://www.niab.go.kr/RDS	C.-D. Han cdhan@nongae.gsnu.ac.kr
Temasek Lifesciences, *Singapore*	Nipponbare	Ac-Ds GT	20 000	3 500	2 000		R. Srinivasan sri@tll.org.sg
EU-OSTID *France*	Nipponbare	Ac-Ds ET	25 000	1 380	1 300	http://orygenesdb. cirad.fr	E. Guiderdoni guiderdoni@cirad.fr
CSIRO Plant Industry *Australia*	Nipponbare	Ac-Ds GT/ET	16 000	611	~50% nonfertile	http://www.genomics.zju.edu.cn/ricetdna	N.M. Upadhyaya narayana.upadhyaya@ csiro.au

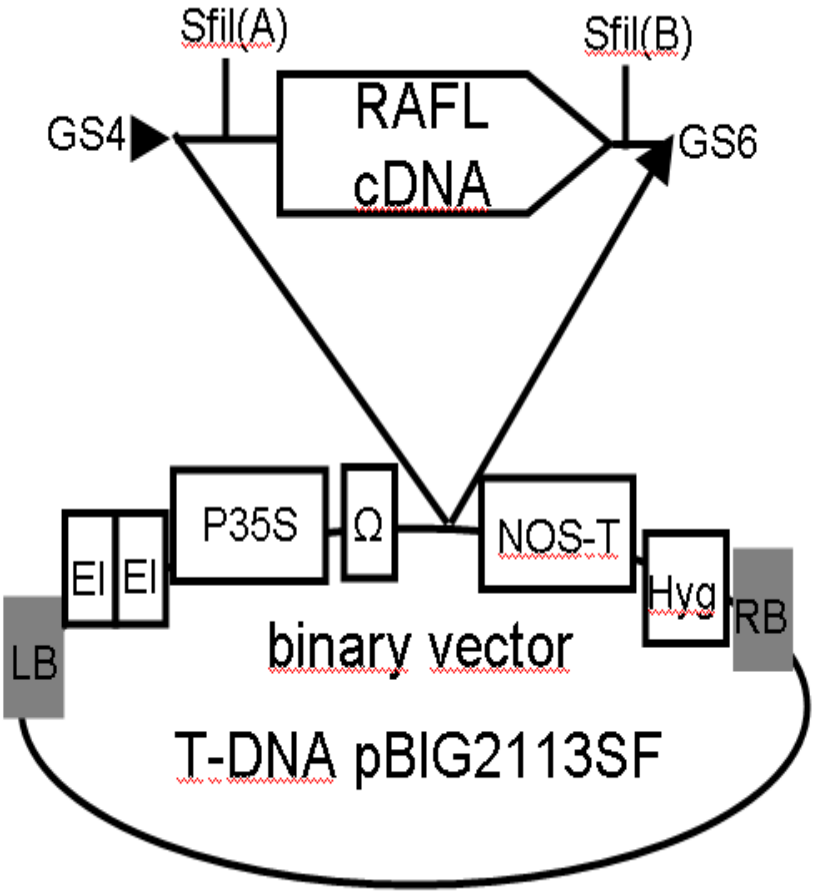

FIGURE 1. The binary vector ***pBIG2113SF*** used in the FOX-hunting system: ***LB/RB*** – T-DNA border sequences; ***EI*** – two tandem repeats of sequence strengthening the transcription (5˘-upstream sequence of CaMV 35S promoter -419 to -90 bp; ***P35S*** – P35S, CaMV 35S promoter -90 to -1 bp; **Ω** – 5'-upstream sequence of TMV, which increases translation effectiveness of inserted gen; ***NOS-T*** – polyadenylation signal of nopaline synthase gen from Ti plasmid; ***Hyg*** – hygromycin resistance gene; ***GS4***, ***GS6*** – sequences of PCR starters to GS4 and GS6 primers used to recover the cDNAs; ***SfiI(A)***, ***SfiI(B)*** – restriction sites for *Sfi*I endonuclease; ***RAFL cDNA*** – **R**IKEN ***A****rabidopsis* **F**ull-**L**ength cDNA

METHODS OF EXPLORATION AND ANALYSIS OF MUTANTS

Brief description of the TILLING-method [36]

TILLING technique combines traditional chemical mutagenesis with the identification of point mutantion using SNPs (**S**ingle **N**ucleotide **P**olymorphism). The critical step in this procedure is in obtaining a sufficiently large population of mutant seed using standard chemical mutagenesis. Following mutagenesis, M1 plants are self-fertilized and the M2 seeds are collected and sown out. DNA from the M2 plants is isolated and used in multiplex PCR reactions.

In order to identify mutations in the gene of interest, gene-specific primers are used to amplify M2 DNA. PCR products are then denaturated and left to cool which causes heteroduplex of unpaired basepairs in the mutation place. By digesting these PCR products with endonuclease *Cel1*, mismatched bases arising from mutations are identifiable after electrophoresis on sequencing gel (Fig. 2).

The classic method of SNP identification bases on multiplying, and then sequencing of selected genes for each individual plant from studied population. The TILLING method of mutant identification is faster, because instead of cost- and time-consuming sequencing, restriction enzyme digests and standard DNA electrophoresis is used. In addition as PCR amplification is multiplexed, and the result of proper analysis accurately indicates the mutant, which not only allows the direct typing for usage in functional studies, but also, in case of plants, allows the immediate use of the mutant in breeding programs.

Brief description of the FOX- hunting [14, 17]

This concept was developed by a team of RIKKEN (Japan) and published under the name Fox-hunting. The strategy has been developed for *A. thaliana* and it is a modification of *Arabidopsis* transformation method by binary vectors that are transferred into plant cells by *A. tumefaciens*.

Essential for the effectiveness of the transformation are sequences of enhancers and transcription terminators, placed between the left and right T-DNA border sequences (LB andRB respectively). Typically, in a FOX-hunting system the binary vectors contains between border sequences, two tandem repeats of transcription amplifier (sequences -490 to -90 bp preceding the promoter of 35S of cauliflower mosaic virus *CaMV*); the 35S promoter (sequence -90 to -1 bp of the *CaM*V virus), Ω leader sequence from the tobacco mosaic virus (which increases the efficiency of translation of the introduced gene), a cassette containing the gene of interest followed by a transcription terminator of the nopaline synthase gene (nos) from the Ti plasmid and finally a gene for resistance to the antibiotic Hygromycin B- a mutant selection gene, between the border sequences. The gene of interest is inserted into the cassette flanked by the GS4 and GS6 primer

sequences which facilitate gene identification in the mutant and contain the recognition sequence for *SfiI*.

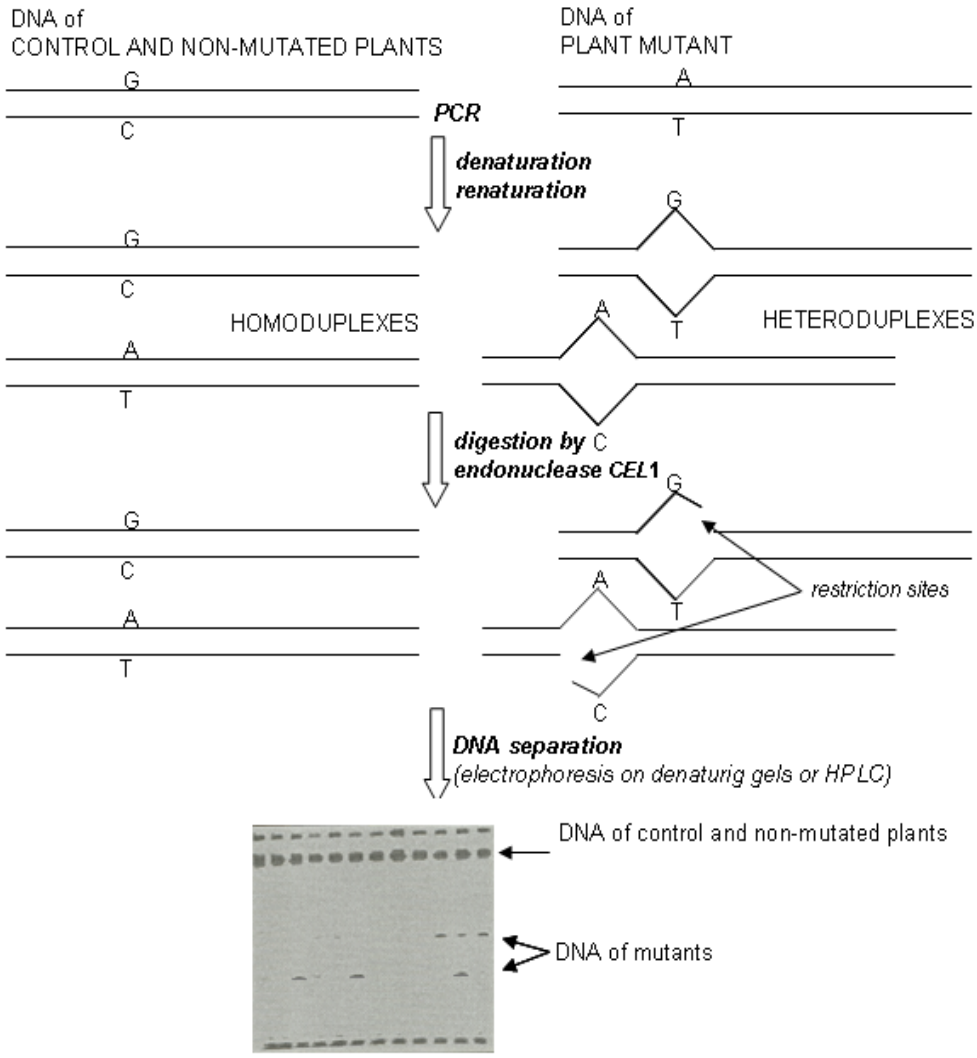

FIGURE 2. Schematic diagram representing the TILLING technique. Equal amounts of mutant DNAs as well as the DNA of the wild plant are mixed and amplified in PCR reaction with gene specific primers. Digestion of that DNA by *Cel*1 endonuclease, specific to unpaired bases of the double-helix DNA, enables mutant identification after the electrophoresis on sequencing gel [10, 13, modified].

In order to generate a library in a binary vector, single copies of each gene collected in cDNA libraries constructed in vectors LambdaZAP and Lambda FLC-1-B are used . In order to standardize of the library, four bacterial genes that cause known phenotypic changes and plants sterility were used. These genes have been cloned at the cleavage site of the restriction enzyme *SfiI*. A mixture of genomic and bacterial cDNA was prepared in such concentration that in mutants bacterial gene expression should be seen with a frequency of 1 / 7500 clones. The mixture was digested with *SfiI*, and then ligated with a modified binary vector pBIG2113N in which the *XbaI* restriction enzyme site include *SfiI* adapters, to enable directional cloning in the "sense" orientation. *Escherichia coli* DH10B was then transformed by electroporation.

While preparing a library is expensive and time-consuming, transformation of *A. thaliana* is relatively straight forward. Once a library has been generated in *A. tumefaciens*, the *Agrobacterium* is resuspended in a 100-200 ml solution containing 5% sucrose and 0, 05% surfactant Silwet L-77. Inflorescences of *A. thaliana* are immersed in this solution for 2-3 seconds, with gentle stirring, so as to cover them with a layer of a suspension of *A. tumefaciens*. After transformation plants are grown in phytotron chamber under elevated relative humidity followed by cultivatation in a standard manner. Seeds harvested from transformed plants are germinated on agar supplemented with Hygromycin B for selection of transgenic plants. Over 15 000 fertile transformants were obtained using this method, which accounts for 77% of the cDNA library. To determine the transformation efficiency 24 lines FOX were tested for the presence of the Hygromycin B resistance using Southern hybridization. All lines tested contained at least one copy of the the Hygromycin B resistance gene with the average number of integrated vectors in the genome of a single line of FOX was 2.6. The average length of DNA inserts in the population of FOX lines was about 30% less than the average length of inserts in cDNA library in *A. tumefaciens* and ranged from 0.2 to 4.6 kb, with a median 1-1,4 kb. A small number of transformants with inserts smaller or larger than the insert in the library in binary vector were detected. The number of transformants with phenotype induced by the bacterial genes was higher than expected, which was explained by differences in multiplication speed of *A. tumefaciens* cells that carried plant or bacterial library. Currently, several FOX-hunting projects for cereal genomes are introduced and implemented throughout the world (Table 5).

Advantages of the FOX-hunting system include the small percentage of co-suppressed genes due to full-length cDNA clones and libraries in the standard binary vectors; a reduction in the number of basic metabolism gene (housekeeping genes) in the standard libraries the facilitation in phenotype analysis which is caused by a short life cycle of *A. thaliana*, and easy isolation and sequencing of genes. The disadvantage of this system is reduction of phenotypic analysis only to genes used for construction of the library in *A. tumefaciens*.

TABLE 5. FOX-hunting projects in studies of cereal genomes

FOX lines [literature]	Genome	Project aim / details	Project Leader
A. thaliana [14, 17]	rice	searches for resistance genes, especially for high temperature; number of mutants: 23 000	Ichikawa Y. youichi@psc.riken.jp
rice [28]	rice	use of the ubiquitin promoter; acquisition and phenotypic analysis of mutants number of mutants: total- 12 000, with single insertion: 8322	Ichikawa Y. youichi@psc.riken.jp
A. thaliana [6]	*Bruguiera Gymnorhiza*	searches for resistance genes against abiotic stresses	Tada Y. tadayui@bs.teu.ac.jp
wheat [39]	wheat	project at the initial stage, small number of stable mutants were obtained	Steber C. jzale@utk.edu

SUMMARY

TILLING and FOX-hunting techniques alow for acceleration of gene functional analysis. Programs that use them can also be an additional source of diversified material used by breeders. TILLING system has been developed to study chemically induced mutants. It enables for immediate and direct introduction of mutants into breeding programs. FOX-hunting system gives new oportunities for functional analysis of genes, generating valuable mutants overexpressing particular genes.

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