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Review Article

Mutagenesis for wheat improvement in the genomics era

Rajender Singh*, Ratan Tiwari, Davinder Sharma, Vinod Tiwari and Indu Sharma
ICAR-Indian Institute of Wheat & Barley Research, Karnal – 132001

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****Corresponding author***

Email: *rajenderkhokhar@yahoo.com*
Tel.: *09466527643*

Abstract

During the last decade, with the unfolding of new fields of genomics, bioinformatics and high throughput technologies for genome analysis, there has been an increased interest in induced mutations within the scientific community of crop improvement. Induced mutations are now being widely used for developing improved crop varieties and for the discovery of genes controlling important traits and understanding the functions and mechanisms of actions of these genes. TILLING (Targeting Induced Local Lesions IN Genomes) represents an extension of the use of spontaneous and induced mutants in plant breeding and allows the direct identification of beneficial genetic changes in genes with known functions and their use as the genetic markers for selection. TILLING has been used for manipulation of starch composition (high amylose and *waxy* starch), grain hardness, high molecular weight gluten subunits (HMW-GS) composition, phytic acid level, disease resistance (rusts, powdery mildew and root pathogens), vernalization and seed dormancy. Because the TILLING population is a permanent resource, the results of basic scientific research can be efficiently translated into crop improvement as new information about the functions of potential gene targets becomes available.

Keywords: Mutagenesis, reverse genetics, functional genomics, TILLING

1. Introduction

Gene discovery and elucidation of gene function through analysis of spontaneous mutations is a well established method. The rate of spontaneous mutations in crop plants is rather low. Mutations arise when base misincorporations or insertion/deletions remain after proofreading by the replicating DNA polymerase, and become established when mismatch repair pathways fail to correct such errors. In addition to replication errors, spontaneous lesions (naturally occurring damage to the DNA) and transposable elements can also generate mutations. However, application of induced mutagenesis through chemicals or radiations increased the frequency of mutations across the genome and generated a vast amount of genetic variability affecting many traits of interest. The pioneering work on artificial induction of mutations was done by H.J. Muller on *Drosophila melanogaster* with X-rays (Muller, 1927). Patterson and Muller (1930) after comprehensive experimental and theoretical analysis concluded that the induced point mutations are changes in the chemical composition of the genes that they may be of varied kinds, and that they probably are, through the possibility of the accumulation of such changes, "endless in their eventual potentialities". Mutants are rare valuable genetic resources in unrevealing the gene functions. The use of mutagenesis in crop breeding has resulted in major advances and the release of more than 2,700 plant mutant varieties (including cereals, pulses, oil crops and ornamental plants) with improved economically important traits (Shu, 2009). Recently, the availability of large amounts of genomic sequences of several plant species led to the development of several reverse genetics tools for functional genomics. The recent developments in reverse genetics and gene discovery technologies have brought a renewed interest in mutation techniques and expanded their use beyond direct application in breeding and classical genetic analysis. TILLING (Targeting Induced Local Lesions INGenomes) is an example of a well-established, almost traditional, mutation breeding method meeting modern technology. It has led to a sea change in enabling isolation of specific mutants in several crops including

wheat (Wang *et al.*, 2012).The range of alleles that can be developed and discovered via TILLING in a short time is unparalleled and unlikely to be found elsewhere in the pool of germplasm accessible to plant breeders (including landraces and undomesticated relatives).

2. TILLING, a reverse genetics tool

TILLING is a reverse genetics tool combining traditional chemical mutagenesis with high-throughput mismatch detection technique to identify series of point mutations within a gene of interest. The method was first developed in *Arabidopsis* to study gene function. Importantly, this method generates a wide range of mutant alleles, is fast and automatable, and is applicable to any organism that can be chemically mutagenized (McCallum *et al.,* 2000).

TILLING platform in plants includes three main steps; creation of mutant population, detection of mutations in targeted sequences and analysis of mutant phenotypes. Mutation can be induced by irradiation with ionizing radiation such as X-rays, gamma rays and neutrons. These physical mutagens often result in large scale deletion of DNA and changes in chromosome structure. By contrast, chemical mutagens most often affect single nucleotide pairs. In plants, widely used chemical mutagens include ethyl methane sulphonate (EMS), methyl methane sulphonate (MMS), hydrogen fluoride (HF), sodium azide, *N*-methyl-*N*-nitrosourea (MNU), and hydroxylamine. The degree of mutation is dependent on the tissue and degree of exposure (dosage × time). Mutations at single nucleotide pairs are generally of most interest to breeders because large-scale changes in chromosome structures usually have severely deleterious results. Usually, a TILLING population consists of 3,000–5,000 M_2 individuals, although larger populations that include 10,000 plants have also been reported. Such populations can serve as a permanent genetic resource of mutations for both forward and reverse genetics.

The determination of PCR 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 desirable to choose a fragment within the coding region. The most potential regions to generate deleterious changes in the targeted gene can be selected with software such as CODDLE (Codons Optimized to Discover Deleterious Lesions) (http://www.proweb.org/coddle/coddle_help.html). Another bioinformatic tool designed for displaying and analysing nucleotide polymorphisms is PARSESNP (Project Aligned Related Sequences and Evaluate SNPs) which determines the effect of single nucleotide polymorphisms (SNPs) on protein, based on the alignment of related proteins(Taylor and Greene, 2003).

The TILLING procedure incorporates the EMS treatment of seeds, M_2 generation, DNA isolation from M_2 plants and pooling, PCR reaction of the fragment of interest, heteroduplex formation and identification of heteroduplexes using denaturing high-performance liquid chromatography (DHPLC) (McCallum *et al.*, 2000). TILLING has been used with many different organisms and many modifications to the procedure have been introduced to automate the screening of mutations at reduced cost. The detection of mutations in the TILLING approach was replaced by the digestion of heteroduplexes using specific endonucleases such as CEL I followed by polyacrylamide electrophoresis (Fig.1) and visualization in the very sensitive LI-COR gel analyser system (LI-COR Biosciences). In polyploid crops such as wheat, the conserved regions of a gene often represent functional domains having high sequence similarity between homoeologous loci. High Resolution Melting (HRM) analysis of mixed PCR amplicons can also detect mutation within three homoeologous genes simultaneously (Dong *et al.*, 2009). Next-generation sequencing (NGS) can significantly accelerate the prospects of identifying mutations at the whole-genome level as the sequencing costs decrease due to improved highthroughput technical accuracy and increased capacity has in TILLING (Tsai *et al.*, 2011).

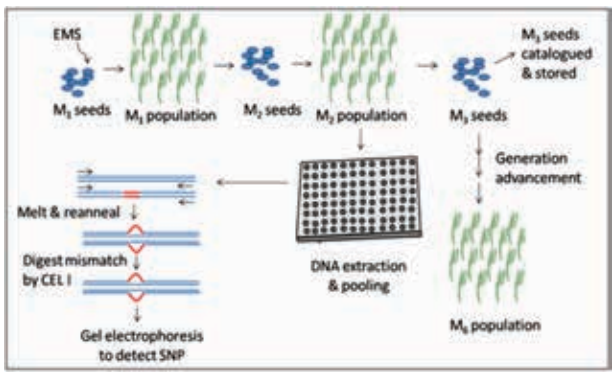

Fig 1. Schematic representation of TILLING technology (modified from Parry *et al.,* 2009)

Prospects of TILLING for wheat improvement

Originally, TILLING was used to investigate gene function in *Arabidopsis* (McCallum *et al.,* 2000) and has since been similarly applied for gene functional analysis in other organisms. The application of TILLING to crop improvement may help to overcome the constraint in domesticated species genomes with limited genetic variation. During domestication and subsequent selection, much of the genetic variation available in the wild crop progenitors has been lost. Thus, plant breeders have at times used wild relatives or landraces

to introduce useful genetic variation. This practice has been successful in wheat for developing disease resistant and higher yielding varieties and a landrace was also used for the development of the first full waxy line because it carried a rare deletion allele of one of the waxy loci (Graybosch, 1998). As an alternative to the use of wild varieties, TILLING can be a means to introduce genetic variation in an elite germplasm without the need to acquire variation from exotic cultivars, thus avoiding introduction of agriculturally undesirable traits. TILLING resources have been developed for numerous crop species. For cereals alone, there are populations for barley (Talame, 2008), rice (Till *et al.,* 2007), and hexaploid and tetraploid wheat (Slade *et al.,* 2005; Dong *et al.,* 2009; Uauy *et al.,* 2009; Feiz *et al.*, 2009; Chen *et al.*, 2012; Bovina *et al.*, 2014). TILLING has been used for manipulation of starch composition, grain processing quality, nutritional quality, disease resistance and several other traits.

3.1 Grain quality: There is now an increasing interest in manipulation of starch composition of wheat due to the recognition of its important role in food and non food application and its use in industry. In addition, research is also focusing on production of high amylose starch because derived foods have increased amount of resistant starch which has beneficial effect on human health (Sestili *et al.,* 2010). Also, production of waxy (amylase free) wheat is also gaining importance because of their use in developing freeze and thaw tolerant grain based food. In wheat, TILLING has been applied to both hexaploid and tetraploid varieties (Table 1.) and was used to create a near null *waxy* phenotype by targeting the gene encoding granule-bound starch synthase (*GBSSI*) (Slade *et al.,* 2005). By intercrossing mutants of the *GBSSI* homoloci produced by TILLING, a complete *waxy* phenotype was produced (Dong *et al.,* 2009). It was also used to identify a hard grain variant in a soft grain background by screening mutants of *puroindoline* (*Pinb*) gene (Dong *et al.,* 2009). TILLING has successfully been used to generate novel alleles for starch-branching enzyme IIa *SBEIIa* genes known to control amylose content in wheat. Single and double null *SBEIIa* genotypes have been found to show a significant increase in amylose (21%) compared to the control content (Botticella *et al.,* 2011). Similarly, double mutant combining a *SBEIIa-A* knock-out mutation with a *SBEIIa-B* splice-site mutation showed 22% increase in amylose content and 115% increase in resistant starch content (Hazard *et al.*, 2012). High amylose forms complexes that resist digestion and mimic dietary fiber (resistant starch). Novel genetic variation in each of the A and B genomes in tetraploid durum wheat and the A, B and D genomes in hexaploid bread wheat have been identified in starch branching enzyme IIa genes (*SBEIIa*). Combining these new alleles of *SBEIIa* through breeding resulted in the development of high amylose durum and bread wheat varieties containing 47-55% amylose and having elevated resistant starch levels compared to wild-type wheat. High amylose lines also had reduced expression of *SBEIIa* RNA, had changes in starch granule morphology and altered starch granule protein profiles as evaluated by mass spectrometry (Slade *et al.*, 2012). The polyploidy nature of wheat is well-suited for TILLING because of tolerance to the high mutation densities. The detection of null mutants is very important in a polyploid species as phenotype of a single mutant is masked by the wild-type homoeologue present in another genome. Because of gene redundancy, it is generally necessary to cross single mutants in A, B and D genome to obtain a functional knockout in wheat (Uauy *et al.,* 2009).

Glu1 locus in wheat contains a pair of tightly linked genes encoding the *x*- and *y*-type subunits which have not been uncoupled using conventional plant breeding strategies. Thus, a specific *x*-type subunit has always been associated with a defined y-type subunit. For example, HMW-GS in the *Glu-D1* locus are usually reported as allelic pairs e.g. *Dx5* + *Dy10*, *Dx2* + *Dy12*. The opportunity to further determine the contribution of the individual HMW-GS subunits to dough quality has been limited due to the unavailability of lines where the *x*-type locus has been uncoupled from the *y*-type locus. *Ax1* and *Dy10* deficient bread wheat lines have been developed using EMS mutagenesis for characterization of their effect on dough mixing properties. The *Dy10* and *Ax1* deficient lines resulted in weaker doughs with faster development time indicating a restructuring of the gluten polymer(Laudencia-Chingcuanco, 2012). The availability of lines deficient in one or more HMW-GS/ LMW-GS in a genotype will resolve the contribution of the individual subunit in dough quality.

High phytate in humans diets significantly decrease the absorption of essential micronutrients such as Ca, Fe, and Zn as phytate forms chelates with these divalent minerals resulting in reduced bioavailability to humans (White and Broadley, 2005). A low phytic acid wheat mutant has been identified from EMS mutagenized M_2 lines of soft white spring wheat. The low phytic acid trait decreased the phytic acid concentration in the bran by 43% and can be used for wheat quality improvement (Guttieri *et al.*, 2004).

Table 1. TILLING populations in wheat and genes/traits studied

Genotype	Species	Size of population	Genes	Mutation densities	Trait	Reference
Svevo	*T. tugidum* L. subsp. *durum*	2601	*SBEIIa-A, SBEIIa-B*	-	Starch composition	Bovina *et al.*, 2014
Jinmai 47	*T. aestivum*	2610	*Ppd-D1* *Rubisco activase A* & *B*	1/34 kb – 1/47 kb	Heading and flowering time	Chen *et al.*, 2012
TA4342-96	*T. monococcum*	1532	COMT1, HCT2, 4CL1	1/92	Lignin biosynthesis	Rawat *et al.*, 2012
Summit	*T. aestivum*	500	*Dy10* & *Ax1*	-	Grain quality	Laudencia-Chingcuanco, 2012
DPW621-50	*T. aestivum*	4,000	-	-	-	Singh *et al.*, 2012
Kronos	*T. tugidum* L. subsp. *durum*	1368	SbeIIa	1/68	Increased amylose content & resistant starch	Hazard *et al.*, 2012
			VRNI, VRN2	-	Amylose content	Chen & Dubcovsky, 2012
			SBEIIa and SBEIIb	1/51	Flowering time	Uauy *et al.*, 2009
Scarlet	*T. aestivum*	2,400	-	-	Grain dormancy	Schramm *et al.*, 2012
		11,970	-	-	Tolerance to *Rhizoctonia solani* AG-8, *R. oryzae*	Okubara *et al.*, 2009
Cadenza	*T. aestivum*	4,500	*Sbe IIa*	1/40 kb	Amylose content	Botticella *et al.*, 2011
		4,500	-	-	Agronomic Bread quality Powdery mildew, rust	Rakszegi *et al.*, 2010
		4,244	*Sgp-1, Wx*		Starch biosynthesis	Sestili *et al.*, 2010
Alpowa	*T. aestivum*	-	-		Broad-spectrum disease resistance	Campbell *et al.*, 2012
		3,000	*Pina*, *Pinb*	1/11.5 kb	Grain texture	Feiz *et al.*, 2009
Ventura	*T. aestivum*	2,348	*Wx-A1, Wx-D1, Pina, pinb*	1/23 kb – 1/37.5 kb	Waxy wheat Grain texture	Dong *et al.*, 2009
UC1041+ Gpc-B1/Yr36	*T. aestivum*	1536	Wheat Kinase Start (WKS)1, WKS2 SBEIIa	1/38	temperature-dependent resistance to wheat stripe rust	Uauy *et al.*, 2009
Express	*T. aestivum*	1152	*GBSS*	1/24	Waxy wheat	Slade *et al.*, 2005
A95631S-Js-12	*T. aestivum*	562	-	-	Low phytate	Guttieri *et al.*, 2004
Guardian	*T. aestivum*	1000	-	-	Thermo-tolerance	Mullarkey & Jones, 2000

3.2 Disease resistance: Wheat leaf rust, stem rust, stripe rust, and powdery mildew caused by the fungal pathogens *Puccinia triticina*, *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, and *Blumeria graminis* f. sp. *tritici*, respectively, are destructive diseases of wheat worldwide. Breeding durable disease resistance cultivars rely largely on continually introgressing new resistance genes, especially the genes with different defense mechanisms, into adapted varieties. A new resistance gene obtained by mutagenesis in wheat mutant from an EMS mutagenized population of the spring wheat cultivar Alpowa with the characteristics of delayed disease development at the seedling stage and complete resistance at the adult plant stage (Campbell *et al.,* 2012). The necrotrophic root pathogens *Rhizoctonia solani* AG-8 and *R. oryzae* cause Rhizoctonia root rot and damping-off, yield-limiting diseases that pose barriers to the adoption of conservation tillage in wheat production systems. Existing control practices are only partially effective, and natural genetic resistance to *Rhizoctonia* has not been identified in wheat or its close relatives. Scarlet-Rz1 was derived from the allohexaploid spring wheat cultivar Scarlet using EMS mutagenesis which showed a high and consistent degree of tolerance to the root pathogens (Okubara *et al.,* 2009).

Other traits

Flowering time is a key component of wheat adaptation and productivity and therefore, a precise understanding of the regulatory mechanisms involved in the initiation of flowering can contribute to develop high yielding varieties adapted to changing environments. Tetraploid wheat TILLING mutants showed that vernalization gene *VRN1* down-regulates the flowering repressor *VRN2* in leaves and is not essential for flowering (Chen and Dubcovsky, 2012). EMS mutagenized population of ABA sensitive genotype Scarlet also resulted in identification of ABA insensitive mutant lines with reduced grain dormancy (Schramm *et al.*, 2012).

Eco TILLING to detect natural variation

Nucleotide variations of important genes in natural populations can also be detected by EcoTILLING, an approach based on TILLING technique. Ecotilling allows the rapid detection of variation in many individuals and is cost effective because only one individual for each haplotype needs to be sequenced (Comai *et al.*, 2004). Novel natural allelic variations in *Rht-1* (Li et al. 2013, *Pinb* (Wang *et al.,* 2008) and *Vrn-A1* (Chen *et al.,* 2011) genes have been detected in wheat by Ecotilling. These examples show the potential of TILLING for wheat improvement. Alleles generated by TILLING can be readily used in traditional breeding programs since the technology is non-transgenic and the mutations are stably inherited.

With the advent of molecular biology, several distinct techniques have been developed to generate or identify mutations to determine the function of genes. Mutagenesis is a powerful tool that establishes a direct link between the biochemical function of a gene and trait of interest. TILLING is a flexible reverse genetics approach that generates a permanent genetic resource that can be utilized to screen multiple traits and genes. TILLING is currently being used for the detection of both induced and natural variation in several plant species. Mutations for important traits or genes associated with disease resistance, tolerance to abiotic stresses, nutritional quality, resource use efficiency etc. can be readily exploited by plant breeders without the legislative restrictions, licensing costs, and society opposition as applied to transgenic approaches.

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