

Review Article

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Tilling: A Tool of Allele Mining in Plants

Suresh^{1*}, Manjeet¹ and Deepak Verma²

¹Department of Genetics & Plant Breeding, ²Department of Zoology & Aquaculture,
CCS Haryana Agricultural University, Hisar (Haryana), India – 125 004

**Corresponding author*

ABSTRACT

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Identification of alleles of a gene and then utilizing them in crop improvement programme is the main objective of any plant breeder. A breeding programme cannot exist if there is no variability. For the screening of germplasm for a desired allele, breeders need large area, labour and fund. Also this screening is amenable to environmental effect. Target Induced Local Lesions In Genome (TILLING) is a perfect solution of these problem. TILLING is a reverse genetics approach which uses mutagens to create noval variability in a particular allele. This variability in a gene is then screened using various sequencers to find out various mutants. The best mutant of them is then can be used in breeding programmes directly. In a modified version, natural population is screened to identify all alleles of a gene. This new version of this technique is called EcoTILLING. Using these reverse genetics approaches, a large number of genes are identified and used in breeding of various crop species.

Introduction

Crop improvement is a science of identification and selection of plants which carry best allele for a gene. So entire crop improvement programmes are based on genetic variability. In a narrow sense we can say that each gene has many alleles which are the result of a wide range of mutations and a plant breeder discriminate between these alleles to find out an allele whose expression is most beneficial for human. This process of studying various alleles of a gene is called allele mining. In the classical breeding, forward genetics approach was used where a large number of individuals were screened for

morphological differences for a particular trait which was thought to have the superior allele of gene governing that trait. But this approach is very cumbersome, time-consuming and labour-intensive. Also functionally defective alleles cannot be identified as these will be unable to express (Gilchrist and Haughn, 2005). Recently with the advancement in large-scale genome sequencing techniques, it is possible to discriminate between different alleles on DNA level. This identification of mutants at DNA level and then finding its effect on morphology of an individual is called reverse genetics. TILLING (Targeting Induced Local Lesions in Genomes) is one of reverse genetics technique which uses

artificial mutagenesis (Koornneef *et al.*, 1982) and sequencing techniques for allele mining (McCallum *et al.*, 2000). It was first used in model plant, *Arabidopsis thaliana*. This technique is based on the fact that a large number of mutants can be created artificially using mutagens (Muller, 1930; Stadler, 1932). Mutations thus produced are identified using sequencing techniques. As the screening is done on DNA level, there is no environmental influence which is a main problem in phenotypic screening

How TILLING is used in allele mining

The whole procedure of TILLING depends on the mutations created by a mutagen. So creation of mutation is the first step of TILLING. As the main target of mutation is specific gene, RNAi or insertional inactivation techniques can be used (Alonso *et al.*, 2003). But these methods are not universal in nature and have some limitations (Que and Jorgensen, 1998). Chemical mutagen, especially ethyl methane sulfonate (EMS) is most widely used because it causes point mutations resulting in single nucleotide polymorphisms (SNPs) (McCallum *et al.*, 2000). These single base alterations may lead to nonsense mutations, missense mutations or silent mutations, thus provide allelic series of mutations of a single gene (Tadele, 2016). Other than mutagen, the experimental material should also be selected carefully. As the purpose of TILLING is to screen new mutants to find out the best allele of a gene, the plant variety used for mutagenesis should be one which is superior for that particular trait. Any plant part which can be used for the propagation of plant can be used. In asexual species, vegetative parts like tuber in case of potato and nodal buds in banana are used. But as these are multicellular, there are problems of chimera formations. To avoid this problem we can use pollens for mutagen treatment because pollens are unicellular cells. But in

majority of TILLING experiments, seeds are used for mutagenesis because their handling is easy. Seeds are pre-soaked in water for some time and then treated with EMS for a particular time. The plants obtained from these mutagenized seeds are M₁ which are selfed to generate M₂. The leaf samples of these M₂ plants are used for DNA extraction using any standard method. DNA extraction using CTAB protocol is most common for most of the plant species (Saghai-Marooof *et al.*, 1984). Equal amount of DNA extracted from these plants is pooled and amplified using PCR. The efficiency of allele detection is higher when pooling is done with DNA of eight individuals. Gene specific fluorescently labelled primers are used for this amplification. This will amplify both original DNA and mutants. The amplified product is denatured using high temperature after which temperature is decreased slowly so that renaturation can occur. As EMS causes point mutation, both original and mutant DNA will be almost complementary, both homoduplexes (between wild type DNA or between same mutants) and heteroduplex (between the wild-type and the mutant strands of DNA) will form. In the next step, amplified products are incubated with CEL1, an endonuclease of the S1 nuclease family which cleave exactly at the 3' side of mismatched base of the heteroduplex without cutting homoduplexes (Oleykowski *et al.*, 1998; Kulinski *et al.*, 2000). Initially, McCallum *et al.*, (2000) used DHPLC, for identification of mutants, where the presence of a heteroduplex was detected as an extra peak in the chromatogram. After the use of CEL1, it becomes more simple and reliable to identify heteroduplexes. After cleavage, fragments are run on agarose gel using electrophoresis and polymorphic fragments are identified and sequenced. Based on the size of the fragments carrying the 5' and 3' fluorescent tags, position of the mutation can be estimated. Using the sequence of mismatched fragments, mutant plants are

identified. From these mutants, best allele is identified and used in breeding programmes.

Eco TILLING

EcoTILLING is a modified form TILLING, first used in model plant, *Arabidopsis thaliana* by Comai *et al.*, (2004). The main difference between these two techniques is that in EcoTILLING, our objective is to uncover genetic variation present in natural population as compared to TILLING, where we have to induce mutations for allele mining. This modified version of TILLING is useful for species which cannot tolerate chemical mutagens. So, in EcoTILLING, we have to select a natural population which is thought to have natural variability for various genes (Comai *et al.*, 2004). As naturally cross pollinated species have higher genetic variability, there is no need create mutant which otherwise may be harmful for the experimenter because most of mutagens are carcinogenic also. The other advantage of this modified technique is that individuals with similar phenotypes are excluded prior to sequencing which reduce cost and time. Also we can detect multiple polymorphisms in a single fragment because CEL I will digest only a small proportion of the heteroduplexes at a single position (Till *et al.*, 2006). But still, this technique is not as much popular as that of original TILLING.

TILLING used in different crop species

TILLING was initially used in *Arabidopsis* by McCallum *et al.*, (2000). In the next year, *Arabidopsis* TILLING Project (ATP) was started by the Comai Laboratory at University of Washington in collaboration with the Henikoff Laboratory at the Fred Hutchinson Cancer Research Center and identified different mutants in this species (Henikoff *et al.*, 2004). Wheat gives positive response to mutagens resulting in 35-40 mutations per kb

(Krasilevaa *et al.*, 2016). This shows that the technique of TILLING can be used in this crop very efficiently. In wheat, Dong *et al.*, (2009) applied a modified TILLING approach which helped in identification of 121 mutants in *waxy* genes, *Wx-A1* and *Wx-D1* and 19 mutants in two *puroindoline* genes (*Pina* and *Pinb*). Similarly, in rice, Hwang *et al.*, (2016) applied gamma ray radiations to mine alleles of membrane transport genes and found five mutants containing SNPs in the coding region of genes *OsAKT1*, *OsHKT6*, *OsNSCC2*, *OsHAK11* and *OsSOS1*. The applicability of induced mutation for creating genetic variability in maize was shown by Till *et al.*, (2004). They used mutagenized pollen for their experiment and identified 11 different genes, obtaining 17 independent induced mutations. Similarly, in other crops also we can use TILLING approach to generate new variability and identification of novel alleles. Subsequently, this technique has been successfully applied to many plant species including maize (Weil and Monde, 2007) and cowpea (Cooper *et al.*, 2008).

In conclusion, TILLING and EcoTILLING are becoming the easy to use gene mining tools for plant breeders. These are quick and more reliable than conventional screening of germplasm. Also by use of mutagens, the chance of identification of useful allele increases. But still there are still limitations, like we cannot use mutagen in all species and the use of mutagens may also cause health hazards. Under such circumstances, EcoTILLING may be a good alternative.

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