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# THE USE OF REVERSE GENETICS APPROACH IN PLANT GENOMICS

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***SUMMARY***

***Mutations are origin of novel variabilities. With developing new methods there are new possibilities for plant breeding for desirable characteristics. The aim of this paper is to introduce a method of reverse genetics, particularly TILLING method, and its potential for future scientific work and creating new improved cultivars for both, quantitative and qualitative characteristics. It can be used for plant breeding and getting new information on molecular level. Mutations in TILLING method are induced with chemical reagent ethyl methanesulfonate – EMS. The function of EMS is to cause mismatch in DNA heteroduplex. TILLING can be used in a numerous plant species. EcoTILLING is the method used for studying natural populations. Both of these methods have advantage for not being trans-genetic, but labour and cost effective.***

***Key-words: TILLING, reverse genetics, DNA, plant breeding***

## INTRODUCTION

Evolution of all species depends on mutations. Exploring full potential of genes is a task for plant breeding. Using the knowledge about gene recombinations can result in creating desirable genotype. With constantly growing human population and decrease of available agricultural areas it is important to produce enough quality food which can fulfil needs for daily caloric and nutritional input. Creating new and improved genotypes is very important task. Today many developed methods can be used for creating a new genetic combination which can lead to increased outputs. Plant breeding uses classical methods of selection and new methods on molecular level from the field of plant genomics. Nowadays plants are descendants of chosen wild and domesticated species with improved traits (Poehlman and Sleper, 1995).

The goal of modern plant breeding is improving our cultivars with recombination of genetic material to gain improvement in traits of interest. Selection brought about increased productivity but also narrowed crop genetic diversity (Able et al., 2007).

In 1980s with the advent of molecular markers, we become able to find and monitor DNA polymorphism responsible for majority of variation in phenotypic traits (Able et al., 2007). The problem is to determine which part of the sequence control changes and how to predict effect on phenotype (Moose and Mumm, 2008). One of the new tools that are given in our hands is reverse genetics which can help us to study genetic variation in a more precise manner. Unlike forward genetics, where we know phenotype and we need to sequence gene to find out it genetic constitution, in a reverse genetics we are aware of gene sequence but its function is uncertain (Tierney and Lamour, 2005).

One of the best developed and explored methods of reverse genetics is TILLING method.

The aim of this paper is to present main features of TILLING method, its potential, caveats and drawbacks in plant genomics and breeding research.

## THEORETICAL BACKGROUND

The term gene was coined by W. Bateson and first appeared at the beginning of 20$^{th}$ century. Maybe the best way to explain gene is a sequence region of DNA that contains information necessary for generating a functional product (Spieth and Lawson, 2006).

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The DNA sequence participates in the process of protein synthesis. This process is usually divided in two stages. The first one is transcription and the second is translation. Transcription starts with transcription factors binding to the promoter region or to enhancers. In some cases gene transcription can be initiated by the activity at an upstream locus control region (LCR). After mRNA is being synthesised it will served as a template for making polypeptide chain in the process of translation. By studying the DNA sequence we can find out their function at phenotypic level. This is the general idea of reverse genetics. The best way to explore what gene action affects is to create small changes in DNA sequence.

Mutations can be created either with insertion or deletion base pairs in DNA sequence. The first is done with inserting a piece of DNA and latter with chemicals (Hardy et al., 2010).

TILLING (Targeting Induced Local Lesions in Genomes) allows us identification of mutation in specific gene. Since mutations give us new features in organisms the discovery of their inducing (Stadler, 1932) was one big step toward the understanding of underlying mechanisms in genetics, especially of higher organisms (McCallum et al., 2000). TILLING method was first used at *Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington* by Claire M. McCallum, Luca Comai, Elizabeth A. Greene and Steven Henikoff in 2000 (McCallum et al., 2000).

The biggest advantage of TILLING method is its capability to identify variation in single base pair (Gilchrist and Haughan, 2005). It allows us to take a closer look in the ATGC sea of billions base pairs.

A TILLING is also closely associated with eco-TILLING. TILLING is based on mutation breeding and reverse genetics whilst ecoTILLING monitor natural diversity and mutations (Backes et al., 2007). With the help of reverse genetics it could be possible to modify specific gene i.e. trait and create the variation needed. This is especially important in plant breeding.

Choosing the best breeding material has been done for years only by observing and measuring its phenotypic values. Today we have opportunity to create plants with single desirable characteristics. These plants are created using transgenic methods. Use of genetically modified organisms in plant breeding is today accepted mainly for maize and soybean. Due to problems with public acceptance and possible scepticism toward genetic transformations, for breeding and research purposes, the principles of reverse genetics are more attractive. All changes are made in the same organism. Mutations are natural and we just observe them and analyze possibilities of their use for improvements.

## METHODS OF REVERSE GENETICS

The methods of reverse genetics are:

a) insertional mutagenesis,

b) gene targeting by homologous recombination,

c) gene silencing and random chemical modifications of the genome and

d) screening (TILLING) (Hardy et al., 2010).

Insertional mutagenesis uses transposones being DNA sequences that can relocate and insert itself from one genomic location to another. The method has been efficient in identifying essential genes for use in animal and plant models (Tierney and Lamour, 2005).

Gene targeting by homologous recombination can be used for gene engineering at specific chromosomal loci. The process is conducted in meiosis with generating crossover exchanges between non-sister chromatids (Hardy et al., 2010).

Gene silencing inhibits expression of a target gene. The process is also known as RNA interference (RNAi). With RNA interference we can analyze phenotype and discover what function of silenced gene is (Tierney and Lamour, 2005).

TILLING method is non-transgenic method which can help us to induce and identify new and existing genetic variation. The advantage of TILLING method is that it is not species specific and can be applied in various organism, as it is in 20 plant species so far (Jankowicz-Cieslak et al., 2011).

TILLING can be used in plant breeding as a source of new mutations. The advantage of the method is that alleles generated by TILLING can be used in selection because mutations are stably inherited (Uauy et al., 2009). The first step is to treat seeds with EMS (ethyl methanesulfonate). EMS is alkylating agent which cause impairing of complementary bases. It alkylates guanine base which then pairs with thymine instead of cytosine (Simsek and Kacar, 2010).

After growing and getting second generation, the next step is PCR reaction. Mutations can be detected by melting and reannealing point. The result of that is heteroduplex DNA with one strand from the mutant and the other from wild-type PCR product (Figure 1). Using specific Cel*I* enzyme mismatch occuring at the point of mutation can be detected, cleaved and then separated by gel electrophoresis (Perry et al., 2003).

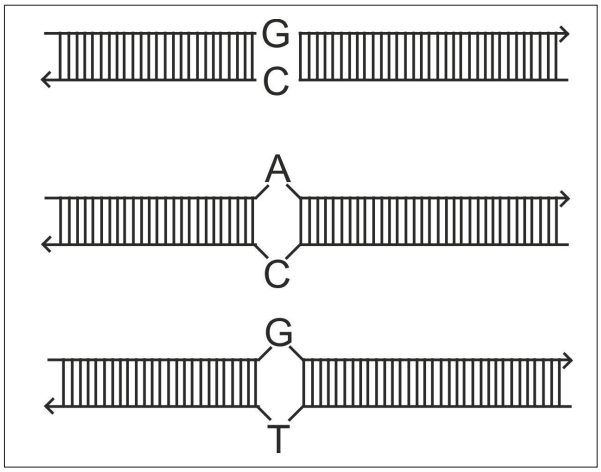


**Figure 1. Difference between normal PCR product and hetero-duplex DNA after EMS mutations (source: http://www.lotusjaponicus.org/tillingpages/heteroduplex.htm)**

*Slika 1. Razlika između normalnog PCR produkta i heteroduplekса u DNA nakon mutacije s EMS-om (izvor: www.lotusjaponicus.org/tillingpages/heteroduplex.htm)*

In the last decade TIILING was successfully used for plant mutations. The method was first used in the experiment with *Arabidopsis thaliana* and then in *Lotus japonicus* (Perry et al., 2003). *The* research was expanded on cultivated crops like rice, wheat and maize, as well. Tilling was tested on a polyploidy species like wheat which is allo-hexaploid in its nature with three different homoeologues genomes. Mutants in polyploid species can be masked with wild-type homoeologue from another genome. For that reason discovering mutants have great value. Over 200 mutations were discovered with 1 mutation/40 kb in tetraploid wheat and 1/24 kb in hexaploid wheat (Simsek and Kacar, 2010).

EcoTILLING is a term used to explain process of detecting polymorphisms in natural populations (Varshney et al., 2006). This method is fast and cheap tool for detecting the spectrum of variations. Information about protein synthesis is contained in the form of triplet of nucleotides called codon which represents single amino-acid. With ecoTILLING we can discover polymorphisms important because of possible effects on codon structure, and thus can have impact on the encoded protein (Comai et al., 2004). The difference between TILLING and ecoTILLING can be seen in Figure 2.

Model plant for ecoTILLING and other genetic research in plants is *Arabidopsis thaliana*. Simplicity of the Arabidopsis genome with only five chromosomes made it useful for scientific projects. Results can then be applied in mass production crops.

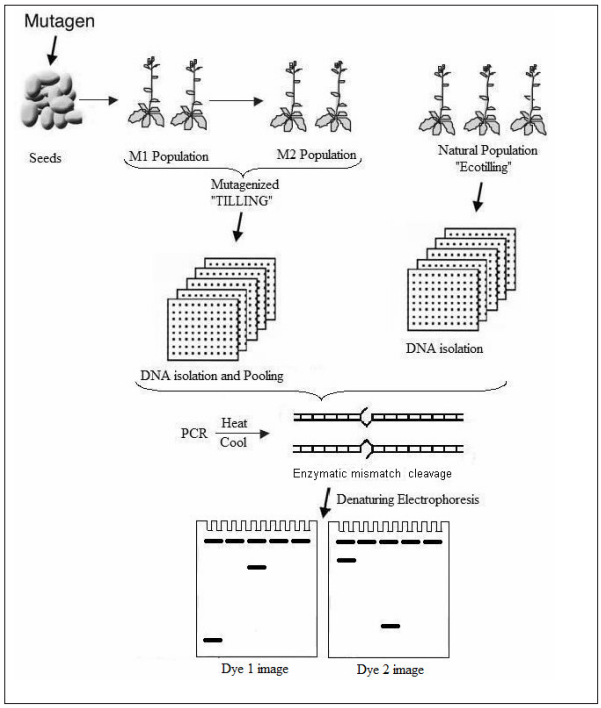


**Figure 2. The difference between TILLING and ecoTILLING procedure (Simsek and Kacar, 2010)**

*Slika 2. Razlika između TILLING i ecoTILLING postupka (Simsek i Kacar, 2010.)*

## DISCUSSION

Domestication and selection through centuries lead to the loss of genetic variation available in natural populations. Using TILLING method we can expand genetic variation and create new combinations (Slade and Knauf, 2005). Some results of TILLING projects are shown in the Table 1.

**Table 1. Results of using TILLING method on plants**

*Tablica 1. Rezultati primjene TILLING metode na biljkama*

| *Organism* | *Common name* | *Mutagen* | *Population size (number of plants)* | *Mutation frequency* | *Reference* |
|---|---|---|---|---|---|
| *Arabidopsis thaliana* | Arabidopsis | EMS | 3712 | 1/89 | (Martin et al., 2009) |
| *Avena sativa* | Oat | EMS | 2550 | 1/38; 1/20; 1/22,4 | (Chawade et al., 2010) |
| *Hordeum vulgare* | Barley | EMS | 4600 | 1/1000 | (Caldwell et al., 2004) |
| *Triticum aestivum* | Hexaploid wheat | EMS | 2348 | 1/23,3 to 1/37,5 kb | (Dong et al., 2009) |
| *Triticum aestivum* | Hexaploid wheat | EMS | 1536 | 1/38 | (Uauy et al., 2009) |
| *Triticum aestivum* | Hexaploid wheat | EMS | 1152 | 1/24 | (Slade et al., 2005) |
| *Oryza sativa ssp japonica* | Rice | EMS | 768 | 1/294 | (Till et al., 2007) |
| *Glycine max* | Soybean | EMS | 529 | 1/140 | (Cooper et al., 2008) |

Source: Jankowicz-Cieslak et al. (2011)

Mutation frequencies shown in Table 1 are high. However, discovering mutations is important as well as inducing them into gene. The first method used was denaturing HPLC (McCallum et al., 2000). This method was replaced by enzymatic mismatch cleavage and fluorescence detection of cleaved bands fractioned by denaturing polyacrylamide gel electrophoresis (Colbert et al., 2001). There are also non-enzymatic techniques: High Resolution Melt (HRM) and Confirmation Sensitive Capillary Electrophoresis (CSCE) (Jankowicz-Cieslak et al., 2011).

The problem with using all of these methods is high cost and need for special equipment. Efforts are required to be taken to develop cost effective method which can be used in most laboratories. The best results so far are with the use of crude celery juice extracts, but comparisons with standard methods in accuracy and sensitivity are still to be carried out (Jankowicz-Cieslak et al., 2011).

## CONCLUSION

Using reverse genetics can get information on plant species on molecular basis. Due to this fact there is possibility of speeding up selection and getting more desirable results. TILLING method can provide us with a new variability and ecoTILLING information on natural mutations in plant organism. This enables making new combinations of crossing and getting field crops with desirable characteristics. Using TILLING has advantage in choosing different methods for analyzing results which puts an option to research work even with lower financial capacity. TILLING is applicable on every plant species and because of that has great potential for usage. New variations gained by TILLING are stably inherited.

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## KORIŠTENJE METODA OBRNUTE GENETIKE U BILJNOJ GENOMICI

***SAŽETAK***

***Mutacije su izvor novih varijabilnosti. Razvojem novih tehnologija, dobivamo mogućnosti u oplemenjivanju bilja za točno određene karakteristike. Cilj ovoga rada je predstaviti metode obrnute genetike, naročito TILLING metodu, te njen potencijal za budući znanstveni rad i stvaranje novih poboljšanih kultivara na kvantitativna i kvalitativna svojstva. Može se primjenjivati u oplemenjivanju bilja zbog dobivanja novih informacija na molekularnoj razini. Postupak se odvija izazivanjem mutacija kemijskim reagensom ethyl methanesulfonatom-EMS. Funkcija EMS-a je u spajanju nekomplementarnih baza u molekuli DNA. TILLING se može koristiti kod velikoga broja biljnih vrsta, uz korištenje naprednih i troškovno prihvatljivih tehnologija. EcoTILLING je metoda koja se koristi za proučavanje prirodnih populacija.***

***Ključne riječi: TILLING, obrnuta genetika, DNA, oplemenjivanje bilja***

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