TILLING: Strategy and application in Crop Improvement

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Introduction

Plant breeders are no longer constrained to transformable species or frequently unreliable forward genetic screenings after the advent of Targeting Induced Local Lesions in Genomes (TILLING). TILLING is a non-transgenic reverse genetics approach which involves mismatch specific restriction enzymes to detect natural or chemically induced mutation in gene of interest. In last two decades the process of TILLINNG has faced a lot of improvement and ultimately inclusion of Next Generation Sequencing (NGS) has made it a paramount of importance in crop improvement. Rapid high throughput TILLING conveniently induces advantageous point mutation irrespective of the genome size or ploidy level or reproduction type of the crop. Even in the era of CRISPR/Cas9 or TALENS, TILLING is also promising as because it allows low cost phenotyping of huge population of plants even before bringing them to the field and it also helps in targeting genome wide multiple genes simultaneously (Irshad *et al*., 2020). However, mutagenesis has been regarded as a safe strategy, particularly in nations with rigorous transgenic legislation. TILLING deals with M2 generation raised by self-pollination of M1 generation mutagenized by Ethyl Methane Sulfonate (EMS), an alkylating chemical mutagen. EMS is extensively used in TILLING as because it can produce a wider range of mutations than insertional mutagenesis (Holme *et al*., 2019).In this article we intended to describe how TILLING has been used to minimize different daunting problems of different crops.

Strategy

Application of EMS can lead to an induction of novel alleles including missense and truncation variants irrespective of genome size and ploidy level of the crops generating M1 population. DNA samples from the M2 generation are pooled together, placed onto microtiter plates, and undergo gene-

specific PCR (Colbert *et al.*, 2001). An endonuclease, such as CELI, a member of the S1 nuclease family of single strand-specific nucleases, is incubated with amplification products. CELI acts by cutting the 3' side of mismatched DNA, specifically at areas where there's a loop formation due to the difference between wild-type and mutant DNA strands and the homoduplexes, remain unaffected (Kulinski *et al.*, 2000). Cleaved fragments are subjected to electrophoresis using an automated gel sequencing device. The resultant gel patterns are then interpreted through commonly-used commercial software for image analysis. This quick assessment method pinpoints the location of the mutations with an accuracy range of ±10 base pairs for PCR fragments that measure about 1 kilobase in length (Henikoff *et al.*, 2004). CAMBa is a specialized tool tailored for identifying variants in TILLING experiments. It utilizes Bayesian statistics to pinpoint the most likely mutations for each individual within a TILLING study (Missirian *et al.*, 2011). Different tools like CODDLE is used for identification of candidate genes or SAS or PARSESNP or SOPMA is used for analyzing the effects of the detective mutations (Slota *et al.*, 2017). EcoTILLING could also result in the natural induction of SNPs and InDels in the genome.

Application in Crop improvement:

TILLING has also been successfully used in different crop plants like rice, wheat, maize, barley, sorghum, oats, pea, tomato, sunflower, Arabidopsis, maize, lotus, barley, potato, soybean, *Brassica oleracea*. *B. rapa,* melon etc. TILLING is also fruitful for allele mining in different vegetables (Selvakumar *et al.*, 2023). Brief updates of crop wise different genes that have been targeted by TILLING have been furnished in the following section.

1. **Arabidopsis:**

One of the pioneer plants where TILLING was initially used was Arabidopsis. TILLING has been employed to develop mutants of CONSTANS (CO) gene which is involved in regulation of flowering time. A truncation mutation in the DUO POLLEN 1 (DUO1) gene that prevents sperm cell development was also discovered (Lai *et al.*, 2012). TILLING made it easy to identify the mutants in the closely linked MEKK2 and MEKK3 gene cluster (MAPK/ERK kinase 1) (Bush *et al.*, 2010).

2. **Wheat:**

A number of wheat genes related to agronomically desirable characters, flour quality or powdery mildew resistance, spike development, carotenoid metabolism, plant height, gluten content etc. have been targeted by TILLING leading to significant outcomes. TILLING induced variants of grain hardness related puroindoline genes, pin a and pin b and waxy gene (Wx) were identified. Again mutants of AGP gene responsible for starch biosynthesis in wheat endosperm were identified by

TILLING approach followed by subsequent function analysis (Guo *et al*., 2017). Through TILLING it was possible to identify new alleles of SBEIIa gene in both winter and Durum wheat leading to elevated amylase and resistant starch content. TILLING has been extensively expedient in describing the role of Cytochrome P450 Monooxygenase in Orobanchol biosynthesis that results in Fusarium Head Blight susceptibility (Changenet *et al*., 2021). Wheat Kinase Start (WKS1, WKS2) genes have also been targeted for TILLING.

3. Rice

Rice genome was mutagenized and then subjected to TILLING to pinpoint different genes related to flowering time, drought resistance, starch synthesis, agronomically important traits or grain quality. Mutations in genes like BZIP (responsible for different biological processes), DREB (Dehydration Responsive Element Binding Protein; stress related gene), PITA (Membrane Receptor Protein responsible for blast disease resistance), R1A (Late blight resistance gene), MAPK (Mitogen-activated protein kinase; growth and development related gene), RPLD1 (Phospholipase related to stress biology, signal transduction, protein trafficking), TPS1 (trehalose-6-phosphate synthase; related to abiotic stress tolerance), EXTE (related to plant growth) were induced and studied for subsequent function analysis (Till *et al*., 2007). Wx (Waxy gene; related to amylase synthesis), ALS (Acetolactate synthase; responsible for herbicide tolerance) have also been mutated by TILLING. Novel allelic variants for low phytic acid, boron toxicity tolerance, salt tolerance, drought resistance of rice have been explored.

4. Barley

In order to find mutants in barley with distinctive characteristics like malting quality, floral parts regulation, fungal infection, TILLING has been extensively used. For instance, genes involved in starch metabolism that affect the malting process were chosen as targets. The phytic acid metabolism-related HvPAPhy_a and HvPAPhy_b genes affecting the nutritional composition of seeds have been targeted for TILLING. BRASSINOSTEROID-INSENSITIVE1 (BRI1) gene for barley height has also been mutated in barley. Genes for chlorophyll protein (Lhcb1), heat shock protein (HSP17.8) have been identified by EcoTILLING.

5. Soybean

TILLING was utilized by soybean researchers to identify gene mutations that affect fatty acid metabolism and alter the oil composition of the seeds for example FAD2, FAD3 (Fatty Acid Desaturase), SACPD gene mutation has led to improved fatty acid metabolism without affecting nodule health. Hypoallergenic soybeans were also developed by employing TILLING. EcoTILLING aided

identification of natural polymorphisms of genes for seed proteins (Gy1, Gy2, Gy3, Gy4, Gy5) has been beneficial in soybean improvement.

6. *Lotus japonicas*

TILLING was exploited on Lotus, a model legume plant, to find mutations in nodulation genes like LjSym2, which is essential for the symbiotic connection between legumes and bacteria that fix nitrogen. TILLING has demonstrated a preference for glycine substitutions in functionally deficient ethyl methanesulfonate alleles. TILLING has confirmed that lack of Nodule-Enhanced Sucrose Synthase does not preclude nitrogen assimilation and fixation in *Lotus japonicus* mutants.

7. *Brassica* sp

BraA.RPL.a, BraA.RPL.b, BraA.RPL.c, BraA.IND.a, BraA.MET1.a and BraA.MET1.b genes orthologous to Arabidopsis REPLUMLESS (RPL) INDEHISCENT (IND) and METHYLTRANSFERASE1 (MET1) have been targeted in *Brassica rapa*. On the other hand Bna.A03. and Bna.C03, genes of *Brassica napus* orthologs of Arabidopsis FATTY ACID ELONGATION1 (FAE1) gene influencing seed oil content have been mutated by TILLING.

8. Pea

TILLING was used to target genes involved in disease resistance and plant architecture. Through this method, a variety of allelic variations were discovered, and they were later linked to particular phenotypes. For example, a gene responsible for N fixation, psSym36 has been mutated.

9. Tomato

TILLING led mutants in genes related to fruit ripening, pathogen resistance, quality improvement has helped in tomato improvement. Mutants of Ripening Inhibitor (RIN) gene related to fruit ripening have been identified by employing TILLING. TILLING was also used to find two SlETR1 allelic mutants (Sletr1-1 and Sletr1-2) that have attenuated ethylene responses.

10. Maize

TILLING aided mutant identification has been successfully utilized in maize for traits such as kernel improvement, chromomethylase etc. Genes related to seed development such as bnlg1861 has been mutated in maize.

11. Melon

Mutants of CmACO1 gene for ethylene biosynthesis identified by TILLING showed differential change in fruit ripening.

12. Chick pea

Mutations of gene related to seed weight, drought, Ascochyta blight resistance etc. have been explored in TILLING populations of chick pea.

13. Lentil

Efforts were made to identify allelic variants that contribute to seed size, quality, disease resistance etc.

Conclusions

It's crucial to bear in mind that the genes listed here only make up just a handful of the plant genes that has been targeted in TILLING. More and more allelic variants will be found as researchers continue to apply TILLING to new plant species and novel genes, advancing our knowledge of gene function and assisting in crop development.

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