UDC 577.2:57.088:635.64

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TILLING-TECHNOLOGY AND FUNCTIONAL MOLECULAR MARKERS IN TOMATO BREEDING (REVIEW)

The information about the powerful new technology TILLING (Targeting Induced Local Lesions IN Genomes) is in the review; the basic procedure and examples of its economically successful using in tomato breeding are described; data on molecular markers of genes / loci related to tomato important agronomic traits are given.

Key words: *TILLING-technology, mutagenesis, molecular markers, tomato*.

Currently nuclear and organelle genomes nucleotide sequences of many plant species are determined including the tomato genome [1–3]. Now, it is important to use the genome sequence information for cognition the gene functions.

In recent years, new emerging technology — TILLING (Targeting Induced Local Lesions IN Genomes) [4, 5] allows systematic functional genomic studies and identification of an allelic series of mutants with a range of modified functions for desired genes [6, 7]. TILLING is a reverse genetic strategy that utilises chemical mutagenesis for inducing variability and sensitive molecular screenings to identify point mutations responsible for phenotype alteration.

Basic TILLING method allows for high-throughput identification of single-base-pair (bp) allelic variations [8]. The first step starts with mutagenized seeds obtained from treatment with chemical mutagen such as Ethyl methanesulfonate (EMS). The resulting M1 plants are selffertilized and the M2 generation of individuals is used to prepare DNA samples for mutational screening. The DNA samples are pooled, arrayed on microtiter plates and subjected to gene-spesific polymerase chain reaction (PCR). Amplification products are incubated with an endonuclease such as CELI, a member of the S1 nuclease family of single strand-specific nucleases. CELI cleaves the 3' side of mismatched DNA where the heteroduplex between the wild-type and the mutant strands of DNA loops out; homoduplexes are left intact. Cleavage products are electrophoresed using an automated sequencing gel apparatus, and gel images are analyzed by examining the gel readout with the aid of a standard commercial image-processing program. Differential doubleend labeling of amplification products allows for rapid visual confirmation because mutations

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are detected on complementary strands and can be easily distinguished from amplification artifacts. Upon detection of a mutation in a pool, the individual DNA samples are similarly screened to identify the plant carrying the mutation. This rapid screening procedure determines the location of a mutation to within ±10 bp for PCR products that are 1-kb in size. For mismatch-specific cleavage, several enzymes, including S1 nuclease and T4 endonuclease VII have been used. CELI, a plant-specific extracellular glycoprotein, has been shown to be suitable for genotyping applications because it preferentially cleaves mismatches of all types and has been used to detect heterozygous polymorphisms in DNA pools. Also, plant extracts performed as well as the highly purified preparations. These results suggest that something in the crude sample either enhances the endonuclease activity of these enzymes at heteroduplex sites or inhibits their activity at the amplicon ends. Screening is performed on DNAs that have been arrayed in 96-well microtiter plates and pooled eightfold to maximize screening efficiency. Gene specific, fluorescentlytagged primers are used to amplify pooled DNA. The amplification products are denatured and allowed to reanneal, generally by heating and cooling. As a result, a mutant strand will often reanneal with a wild-type strand, creating heteroduplexes at the site of the mutation or polymorphism. The resultant double-stranded products are digested with CELI, which cleaves one of the two strands at the heteroduplex mismatches. Cleaved products, which are detected on polyacrylamide denaturing gels, identify individuals that have a mutation in the gene of interest. The size of the fragments carrying the 5’ and 3’ fluorescent tags can be used to estimate the position of the mutation within the amplicon (fig.).

So, TILLING is a method that allows directed identification of mutations in a specific gene and combines a standard and efficient technique of mutagenesis with a chemical mutagen with a sensitive DNA screening-technique that identifies single base mutations (also called point mutations) in a target gene. The only prerequisite for its application is the knowledge of the gene nucleotide sequences.

Tomato (*Solanum lycopersicum* L.), ranking first in the world for vegetables, is important crop with numerous uses as fresh and processed produce and with a high nutritional value. It is also acknowledged model specie for research on fruit development and metabolite accumulation. For major goals of tomato breeders (higher productivity, better tolerance to biotic and abiotic stresses and increased sensory and health value of the fruit) targeted mutations in genes connected to traits of interest creating knockouts or modified activities may provide useful material.

For tomato some mutant populations are created on different genetic background: EMS and fast-neutron mutant populations — cultivar «M82» [10]; EMS population — cultivar «Red Setter» [11]; EMS population — cultivar «TPAADASU» [12]; EMS and γ-ray-irradiated populations — cultivar «Micro-Tom» [13]; EMS populations — cultivars ‘Best Of All’ and ‘Money Maker’ [14]; EMS and N-methyl-N-nitrosourea (MNU) populations — cultivar «Heinz 1706» [15].

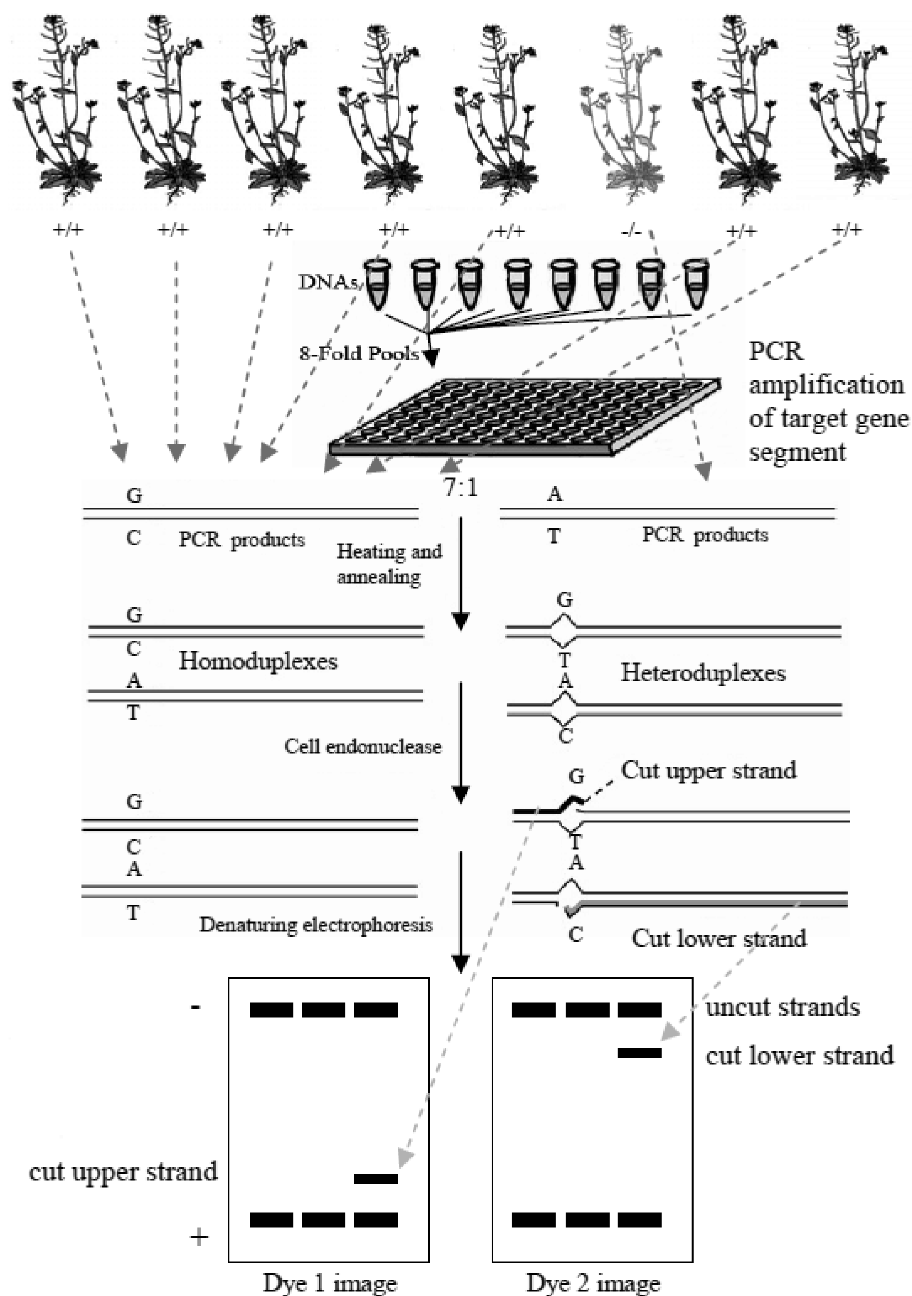

Fig. Schematic diagram of TILLING method [9]

In tomato, four independent TILLING platforms that consist of variety of genetic backgrounds (i.e., M82, Micro-Tom, Red Setter and TPAADASU) have been developed by different groups.

M82 TILLING platform — 4759 M3 families mutagenised at 0,7 % EMS. More than 80 genes screened (eIF4E, DET1, COP1-like, DDB1a, COP10, NAM, ACO1, E8, DHS, Rab11a, PG, MET1, Exp1 CRTISO, CUL4 *etc*). Mutation frequency — 1/574 kb. Phenotypic database was developed [16].

TPAADASU TILLING platform — 8225 mutant lines treated with 1,0 % EMS. Mutation frequency — 1/737 kb. Screened gene — ARF7, ProDH, PSY1, Sus2.

Red Setter TILLING platform. To apply TILLING to tomato, a new mutant collection was generated in the genetic background of the processing tomato cultivar Red Setter by treating seeds with two different EMS doses (0,7 % and 1,0 %, mutation frequency — 1/574 kb, 1/322 kb). An associated phenotype database, LycoTILL, was developed [17] and a TILLING platform was also established. The interactive and evolving database is available online to the community for phenotypic alteration inquiries. To validate the Red Setter TILLING platform, induced point mutations were searched in 7 tomato genes, involved in fruit quality traits, with the mismatch-specific ENDO1 nuclease: ripening-inhibitor (RIN) and green ripe (Gr) genes involved in the ripening of tomato fruit, rab11 GTPase (Rab11a) and expansin 1 (Exp1) genes related to the tomato softening control, polygalacturonase (PG) gene involved in the cell wall hydrolysis, and lycopene beta cyclase (Lcy-b) and lycopene epsilon cyclase (Lcy-e) involved in the carotenoid biosynthesis pathway. In total 9.5 kb of tomato genome were screened and 66 nucleotide substitutions were identified. The overall mutation density was estimated and it resulted to be 1/322 kb and 1/574 kb for the 1,0 and 0,7 % EMS treatment respectively. Comparison of this collection mutation density with other TILLING populations demonstrates that the Red Setter genetic resource is suitable for use in high-throughput mutation discovery.

Micro-Tom TILLING platform. DNA pools were constructed from 3052 mutant lines treated with 0,5 or 1,0 % EMS. The mutation frequency was calculated by screening 10 genes. The 0,5 % EMS population had a mild mutation frequency of one mutation per 1710 kb, whereas the 1,0 % EMS population had a frequency of one mutation per 737 kb, a frequency suitable for producing an allelic series of mutations in the target genes. The overall mutation frequency was one mutation per 1237 kb, which affected an average of three alleles per kilobase screened. To assess whether a Micro-Tom TILLING platform could be used for efficient mutant isolation, six ethylene receptor genes in tomato (SlETR1–SlETR6) were screened. Two allelic mutants of SlETR1 (Sletr1–1 and Sletr1–2) that resulted in reduced ethylene responses were identified, indicating that our Micro-Tom TILLING platform provides a powerful tool for the rapid detection of mutations in an EMS mutant library. This work provides a practical and publicly accessible tool for the study of fruit biology and for obtaining novel genetic material that can be used to improve important agronomic traits in tomato.

Using TILLING, Arcadia Biosciences has generated 220 induced mutations in 19 tomato genes implicated in fruit shelf life [18]. These shelf life genes represent many functional protein classes, including cell wall disassembly enzymes. One such gene is the cell wall enzyme, expansin. Antisense reduction of the tomato expansin EXP1 gene increases fruit firmness, reduced biomass

accumulated a fungal pathogen, *Botrytis cinerea* when expansin expression is suppressed. TILLING was used to identify 9 SNPs in our mutagenized library, including 2 nonsense and 7 missense mutations. Early results show increased fruit firmness in a knockout expansin line, similar to the results seen in the transgenic antisense expansin line.

The characterization of natural recessive resistance genes and *Arabidopsis* virus-resistant mutants have implicated translation initiation factors of the eIF4E and eIF4G families as susceptibility factors required for virus infection and resistance function. To investigate further the role of translation initiation factors in virus resistance Piron et al. [19] set up a TILLING platform in tomato, cloned genes encoding for translation initiation factors eIF4E and eIF4G and screened for induced mutations that lead to virus resistance. A splicing mutant of the eukaryotic translation initiation factor, S.l_eIF4E1 G1485A, was identified and characterized with respect to cap binding activity and resistance spectrum. Molecular analysis of the transcript of the mutant form showed that both the second and the third exons were miss-spliced, leading to a truncated mRNA. The resulting truncated eIF4E1 protein is also impaired in cap-binding activity. The mutant line had no growth defect, likely because of functional redundancy with others eIF4E isoforms. When infected with different potyviruses, the mutant line was immune to two strains of *Potato virus Y* and *Pepper mottle virus* and susceptible to *Tobacco each virus*. Mutation analysis of translation initiation factors shows that translation initiation factors of the eIF4E family are determinants of plant susceptibility to RNA viruses and viruses have adopted strategies to use different isoforms.

TILLING has been applied to produce a series of mutations in genes encoding essential components of the tomato light signal transduction pathway in an attempt to enhance fruit nutritional quality [20]. Point mutations to DEETIOLATED 1 (DET1), which is responsible for the high pigment2 (*hp2*) tomato mutant, resulted in elevated levels of both carotenoid and phenylpropanoid phytonutrients in ripe fruit, whilst immature fruit showed increased chlorophyll content, photosynthetic capacity and altered fruit morphology. Furthermore, genotypes with mutations to the UV-DAMAGED DNA BINDING PROTEIN 1, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and COP1like were also characterised. These genotypes largely did not display phenotypes characteristic of mutation to light signalling components but their characterisation has enabled interrogation of structure function relationships of the mutated genes.

So, the strength and potency of TILLING strategy has been validated by its successful application in both animals and plants, including tomato. Induced mutations can then be used as molecular markers to rapidly introgress the trait into any cultivar. TILLING can reveal potentially useful mutations for molecular breeding, inferring introgression lineage or identifying haplotypes.

Molecular markers. Genetic collections creation, identification and preservation have great importance for improving the efficiency of breeding

and genetic research. The tomato mutant forms collections is unique tool for solving theoretical and practical problems of breeding (range of genetic variation available, development of methods of gamete (pollen) selection for resistance to abiotic and biotic environment factors, introduction into breeding lines of genes controlling economically valuable traits *etc*) [21].

Modern plant breeding process combines methods of molecular genetics and traditional selection techniques. Marker assisted selection (MAS) is selection of organisms for traits of interest, such as color, meat quality, or disease resistance, by indirect analysis of genes that control traits of interest with molecular markers. Since 1980s molecular markers are being widely used as a principal tool for breeding of many crops, among these — tomato. Gene pyramiding is a useful approach to maximize utilization of existing gene resources. MAS is an effective approach for pyramiding genes or quantative trait loci (QTLs) from different sources and for different traits into elite germplasm.

Molecular markers have been used extensively for genetic mapping as well as identification and characterization of genes and QTLs for many agriculturally important traits in tomato. Identified and mapped tomato genes and QTLs can be divided into several groups: — disease (fungal, bacterial, viral, and nematode) and insect resistance genes and QTLs; — abiotic stress resistance genes and QTLs (salt tolerance, cold tolerance (low temperature), drought tolerance); — genes and QTLs for flower- and fruit-related characteristics (fruit size, fruit shape, fruit color, fruit soluble solids, fruit yield, fruit ripening). Summary of most genes and QTLs which have been identified and mapped in tomato chromosomes during the past two decades is presented in review article [22].

Different types of molecular markers such as RFLPs, AFLPs, SSRs, CAPS, RGAs, ESTs, SNPs and COSs have been developed and mapped onto the 12 tomato chromosomes. The expanding tomato EST database, the new microarray DNA chips, and the genome sequence data are expected to aid development of more practical markers. Several BAC libraries have been developed that facilitate map-based cloning of genes and QTLs. Sequencing of the euchromatic portions of the tomato genome is paving the way for comparative and functional analysis of important genes and QTLs.

Functional PCR-markers make it possible to diagnose the allele structure of studied forms at different stages of development that accelerates the selection process and increases its efficiency. Known traits for which marker-assisted selection and breeding are done in tomato.

Molecular markers linked to desease resistance gene. More 40 genes (including many single genes and QTLs) that confer resistance to all major classes of plant pathogens have been mapped on the tomato molecular map. In the early 1980s, many tomato seed companies took advantage of the reported linkage association between nematode resistance and *Aps-1* locus [23] and used the *Aps* marker for selecting for nematode resistance. Now

marker-assisted selection and breeding are done in tomato for such pathongene resistance traits: bacterial canker — 3 QTLs, *Rcm2.0, Rcm5.1*, bacterial speck — *Pto*, bacterial spot — *Rx-3*, bacterial wilt — 2 QTLs, blackmold — few QTLs, corky root rot — *Py*-1, Fusarium wilt — *I-2C, I-3*, late blight — *Ph-3*, 4 QTLs, powdery mildew — *Lv, Ol-1, Ol-2*, root-knot nematode – *Mi,* Tomato spotted wild virus – *Sw-5,* Tomato yellow leaf curl virus — few QTLs, Tobacco mosaic virus – *Tm-2a,* Verticillium wilt – *Ve.* Genotyping tests are developed for tomato pathogene resistance trait by markers: Tm-2(a) for Tobacco mosaic virus, Mi-1 and Mi-23 for *Meloidogyne* spp. Nematode (root knot), I-2 and I-3 for *Fusarium oxysporum* f. sp. *Lycopersici* Fusarium wilt, races 2 and 3, SW-5 for Tomato spotted wilt virus, Cf-23 for *Cladosporium fulvum* Leaf mould, Ve for *Verticillium* Verticillium wilt, Pto for *Pseudomonas syringae* pv. *Tomato (Pst)* Bacterial speck, Ty-1 and Ty-3 for Tomato yellow leaf curl virus, Lv for *Leveillula taurica* Powdery mildew, Py-1 for *Pyrenochaeta lycopersici* Corky root rot.

Publically-available molecular markers for major disease resistance traits in tomato and assess their current and potential use in public and private tomato breeding programs are presented in review [24]. The review indicated that although markers have been identified for most disease resistance traits in tomato, not all of them have been verified or are readily applicable in breeding programs. For example, many markers are not validated across tomato genotypes or are not polymorphic within tomato breeding populations, thus greatly reducing their utility in crop improvement programs. Authors discuss markers actual use in tomato breeding programs; many of the available markers may need to be further refined or examined for trait association and presence of polymorphism in breeding populations. However, with the recent advances in tomato genome and transcriptome sequencing, it is becoming increasingly possible to develop new and more informative PCR-based markers, including single nucleotide polymorphisms (SNPs), to further facilitate the use of markers in tomato breeding. It is also expected that more markers will become available via the emerging technology of genotyping by sequencing.

Molecular markers are using for identification of pathogens resistance genes alleles/variants that induced by mutagenesis. Different mutagenesis methods (EMS-chemical, fast neutrons, transposons, T-DNA) induce point mutations, deletions, insertions. In reverse genetics screens, a target gene is chosen and the mutant population is screened for mutation in this gene using the appropriate detection methods [25–28].

Molecular markers of abiotic stress resistance genes and QTLs. In tomato significant efforts have been devoted to identification and mapping of QTLs conferring tolerance to environmental stresses such as salinity, drought and low temperatures, and less research has been conducted on other stresses, including high temperatures. Some QTLs (3–10) have been identified for salt, cold, drought tolerance during seed germination, vegetative growth and later stages in tomato for such traits: root ammonium uptake,

water use efficiency, shoot wilting, ion accumulation etc. It was found that some genes affect tomato seed germination under different stress conditions while other genes are more specific.

Molecular markers of genes controlling the fruits quality. Molecular markers have been used to map genes or QTLs for many flower- and fruit-related characteristics in tomato, including exerted stigma, petal and sepal characters, fruit size, shape, color, soluble solids content, pH, lycopene, acidity, flavor, ripening and others.

Such genes / QTLs identified for traits: fruits ripening — rin (ripening inhibitor), nor (non-ripening), nor4 (alcobaca); growth habit — *sp* (self pruning); carotenoid biosynthesis — *B* (β-carotene), *og* (old-gold), *ogc* (old-gold crimson), *t* (tangerine). Genes *lp, hp, hp-1, hp-1w, hp-2, hp-2j, hp-2dg, dg* associated with increased levels of carotenoids, *r, at, sh* — with low content of carotenoids, *Del, B, t, gs* alter the composition and the ratio of carotenoids. Traditional genetic studies had identified several genes controlling fruit shape in tomato such as *pr* (pyriform), *o* (ovate), *bk* (beaked tomato), *n* (nipple-tip tomato), *f* (fasciated), *lc* (for locule number). Also genes or QTLs for numerous other flower- and fruitrelated characteristics — self incompatibility, unilateral incongruity, transgressive segregation, selfpruning (determinate type plants), jointless pedicel, seed size and number — have been identified. Genes and QTLs that have been identified and/or mapped on tomato chromosomes for such characteristics summarizes in Foolad's review [22].

For example, 45 tomato cultivars and breeding lines collection was studied for presence of mutant alleles of the genes *B*, *og*, *ogc*, *t*, determining carotenoid biosynthesis; *rin*, *nor*, *nor4*, determining fruits ripening; *sp*, determining growth habit with application of functional PCR-based markers [29]. Based on results of DNA-genotyping, the accessions carrying valuable alleles were selected and further used for introduction to breeding process. On the basis of realized studies there was studied the identification of allelic composition of genes controlling the biosynthesis of carotenoids and fruit ripening. A comprehensive, multi-generation, allele test suggests that the tomato mutations dark-green (*dg*) and high pigment 2(j) (*hp-2j*) are allelic [30]. The hp-2(j) mutant is caused by a mutation in the tomato homolog of the DET1 gene, involved in the signal transduction cascade of light perception and morphogenesis. DET1 locus polymorphism was used to develop PCR-based DNA marker, which enables an early genotypic selection for breeding lycopene-rich tomatoes. The results strongly support the hypothesis that tomato *dg* mutation is a novel allele of the tomato homolog of the DET1 gene.

Molecular markers of genes / QTLs for other flower- and fruitrelated traits. Genes and QTLs have been identified for numerous other flower- and fruitrelated characteristics as well as traits such as self incompatibility, unilateral incongruity, transgressive segregation, selfpruning (determinate type plants), male sterility, jointless pedicel. QTLs mapped for such traits branch, bud type, curly leaf, days to emergence, to first flower, to first ripe

fruit, to third leaf, for 'flower' — number/plant, number/truss, node number, for «leaf» — length, for «leaflet» — apex angle, distance/length ratio, length, number, surface area, width, width/ length ratio; node number; for «plant' — cover, fresh mass, growth habit, height; for «seed» — number, weight, size. Such, genotype *pistillate* (*pi*) was characterized that directly recalls mutations affecting class B MADS-box genes [31]. *pi* mutation deserves a particular interest, producing four-whorled, although modified, flowers useful to study the functional linkage between flower induction and flower organ identity specification. The recessive mutation *ps-2*, which appeared spontaneously in tomato, confers functional male sterility due to non-dehiscent anthers. *PS-2* gene was isolated and characterized [32]. This is the first functional sterility gene isolated in the *Solanaceae* family. The role of *Lanceolata* gene mutation in leaf forming with tomato-plants has been investigated [33]. Connections between endoge-nous hormones content in leaves of various tiers, differing in liaf division level have been discussed.

So, using the latest achievements in fields of genetics, molecular biology and biotechnology, breeders can create new varieties with improved useful traits. Introduction of DNA markers, especially those PCR-based has led to breakthrough in the plants genetic research, including tomato. They are successfully used for plant genomes mapping, phylogenetics studies, selection of parental forms in plant breeding, and above all to identify the genes of important traits. For tomato have been identified and mapped 9309 molecular markers [34]. High-density genetic maps development gives an opportuni-ty to use them in genetic research and breeding programs. Identification of DNA markers closely linked to studied gene can significantly facilitate the identification of desirable traits in material breeding, or accelerate the plants selection for elimination of genotypes with undesirable genes [35]. Material breeding selection using molecular markers, defined as MAS is increasingly being used in tomato breeding programs, contributing to facilitated identification of genes or QTLs and their transfer into the cultivated species from wild form.

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Receive 14.03.2014.

УДК 577.2 : 57.088 : 635.64

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TILLING-ТЕХНОЛОГІЯ ТА ФУНКЦІОНАЛЬНІ МОЛЕКУЛЯРНІ МАРКЕРИ В СЕЛЕКЦІЇ ТОМАТА (огляд)

В огляді подається інформація щодо нової потужної технології спрямованих індукованих локальних змін у геномах — TILLING-технологія (Targeting Induced Local Lesions IN Genomes), описана базова процедура, наведені приклади її економічно успішного використання в селекції томата; вміщені також дані щодо молекулярних маркерів генів/локусів, пов’язаних з важливими агрономічними ознаками томата.

УДК 577.2 : 57.088 : 635.64

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TILLING-ТЕХНОЛОГИЯ И ФУНКЦИОНАЛЬНЫЕ МОЛЕКУЛЯРНЫЕ МАРКЕРЫ В СЕЛЕКЦИИ ТОМАТА (обзор)

В обзоре представлена информация о новой мощной технологии направленных индуцированных локальных изменений в геномах — TILLING- технология (Targeting Induced Local Lesions IN Genomes), описана базовая процедура, приведены примеры ее экономически успешного использования в селекции томата, а также представлены данные о молекулярных маркерах генов/локусов, связанных с важными агрономическими признаками томата.