THE CONSTRUCTION OF THE DNA MARKER FOR THE GENE OF TOMATO RESISTANCE TO FUSARIUM WILT

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Abstract

Using GeneDoc software more polymorphic DNA regions have been identified on the basis of the known homologous sequences of the cluster 12. The primers for amplification of the sequences have been designed in accordance with the information obtained. On the whole, 11 primer pairs combined with 20 restrictase patterns have been analyzed. One of the tested combinations turned out to be usefid in distinguishing the resistant plants from the non-resistant ones, as well as for revealing heterozygotes. The DNA marker for the gene of Fusarium wilt tomato resistance developed on the basis of such a combination may be of great value for plant breeding in order to examine genotypes resistant to Fusarium.

Introduction

Fusarium wilt is known to be a disease affecting most crop species thus resulting in great economic losses. Resistant cultivars have been developed not for all crops, the resistance being overcome by new pathogen races rather rapidly.

Fusarium oxysporum f sp. Lycopersici is a soilbome fungus causing tomato wilt disease [1]. The pathogen infects a plant by penetrating into it directly or through wounds. Afterwards the pathogen develops dramatically in the plant xylem tissue, which may result in the plant death in a week after infection. To date, the three fungi races and the corresponding resistant genes have been identified. The locus / responsible for the resistance to race 1 was mapped to the chromosome 11 and transferred from *Solarium pimpenellifolium* in 1939 [2]. The locus *13* introgressed from chromosome 7 of *L.pinnellii* confers resistance to races 1, 2 and 3 [3]. The locus *12* is of great importance for breeding of F] tomato hybrids as it ensures resistance to the most wide-spread races 1 and 2. Moreover, the above mentioned locus was transferred from wild tomato species *Solarium pimpenellifolium* and mapped to the short arm of chromosome 11.

Tomato breeding for Fusarium resistance is known to be a time- and labour-consuming procedure, for it requires tests in the artificially infected plots. Recently, the molecular markers have been applied more frequently due to the advances in the molecular biology techniques, which enables to decrease total costs including labour expenses and facilitate the breeding process. Being carried out with the application of the molecular markers the breeding procedure is known as *markers assisted selection* (MAS). The marker should be cheap and easy to apply, tightly linked to the target trait and co-dominant, that is it should be able to reveal the latent recessive alleles in heterozygotes. It was found that CAPS (cleaved amplifies polymorphism sequence) markers distinguishing the nucleotide differences in the restriction sites meet the above mentioned requirements [6].

In late 1990s, the genes *I2C-1* and *I2C-2* belonging to the cluster *12* conferring resistance to Fusarium wilt were cloned and characterized [2]. According to the further investigations, these genes failed to confer the complete resistance in tomato plants. The gene *I2C-1* turned out to be the only one demonstrating partial resistance. In 1998 one more gene conferring the complete resistance in the plants was cloned from the cluster

and designated as *12*. The gene product was shown to be highly homologous to the previously cloned genes. The subsequent research revealed that the locus *12* consisting of seven genes-homologs is approximately 90 kbp in size [7]. To construct the efficient marker for the resistance gene *12* turned out to be a great challenge for a number of reasons such as the complex structure of the resistance locus *12,* the great number of homologs and the lack of information on the nucleotide sequence of homologous loci for the susceptible plant forms.

The aim of our research is to construct the efficient DNA marker for the gene *12* of tomato resistance to Fusarium wilt in order to use it in plant breeding.

Materials and methods

Plant material. TA number of resistant and susceptible tomato cultivars as well as hybrids have been analyzed. The former were represented by Amparo, DRW, Ildico, Megana, Duraso while the latter by Pitenza, Beliy Naliv, Bruinsma, G7861, G10610. The segregating for the resistance tomato population tested by artificial infection was used to estimate the efficiency of the markers constructed. The population was provided by PhD G.F. Monakhos, the head of N.N. Timofeev Vegetable Breeding Station. The plant materials were obtained from seeds germinated in the Petri dishes at 24°C and grown in the film greenhouse.

DNA extraction. The DNA was extracted from the young leaves according to Bematzky и Tanksley (1986) with certain modifications. The young leaves were ground in the extraction buffer (0.35 M sorbitol, 100 mM Tris-HCl, 5mM EDTA, pH 7) at cool temperature. After centrifuging the tubes at 14 000 rpm for 10 min. the supernatant fluid was removed, then the extraction buffer was added and the tubes were shaken intensively. The subsequent lysis was performed at 65°C in the lysis buffer (1M Tris-HCl pH 7.5, 0.5 EDTA, 5M NaCl, 2% CTAB) with 5% sarcosyl added. Thereafter the tube content was purified by chloroform-isoamyl alcohol (24:1) mixture and centrifuged at 14 000 rpm for 10 min. Then the supernatant was pipetted off and the DNA was precipitated in one volume of isopropanol. Afterwards the tubes were centrifuged at 14 000 rpm for 10 min. and the precipitation was dried and dissolved in MQ-water. The amount of the DNA extracted and the degree of the RNA contamination were estimated by electrophoresis in 1% agarose gel.

Polymerase chain reaction. The PCR mixture (25 |JI) contained: 70 mM Tris-HCl, pH 8,6 (25°C), 0,001% Triton X 100, 16,6 mM $(NH_4)_2S0_4$, 2,5 mM Mg Cl_2, 0,25 mM of each dNTP (Sileks M), 0,5 pM of each primer, 100-150 ng DNA and 1 unit Taq-polymerase (Sileks M, Moscow). The following selected primers were used:

LR1: ACT CTA CAA ATC TGG AAT TTC CTT
LR2: AAA TTC TAG TAG TGG TGT GA
V127: TGG AAC AAT GTC GGC ACT TAT CTT
VU127: CAA CCA CAT TTT CCA ACT TCA CAA
C1: CCT CCT TTT CTC ACC TCA CTT CGC
C2: ATT TGT GGC CAG TAT TCC CC
FG1: 5' ATG GAG ATT GGC TTA GCA GTT GGT 3'
LF127: 5' TCG TTG TAA TTT TCA TTC CAC ACA 3'
LF79: 5' CAT TTC TCA ATT CAT CCC ACT CGT 3'
LF78: 5' TCA TTC CAC ACG TCA TCA AGG ACA 3'
F7 : 5' CAG TTG TGA AGT TGG AAA ATG TGG 3'
FU7: 5' CAA TTT CAA TTT TGG GCA ACG AGA 3'
L79 : 5' ATT TAG GCA ACC AAT TCT TTC TCG 3'

LU79: 5' TGG GCG TAG CTC ATC AAG TAT GTA 3'
M78: 5' TCT TGT TGT CCT TGA TGA CGT GTG 3'
MU78: 5' CTA TTG AAT AGG ACA TGT GCC GAC 3'
U127 5' GTT GAG GAC ATT GCT TCC GAT ACG
UN127 5' AAC AAC TGG ACA ATC ACT AAC TTC
U78 5' ACT CGA TAA AGT ATT GGT TAC CCG
UN78 5' TCA GCT ACA AGT CAA ATT GAA GGC
UP78 5' TGT ATT GAT TAT ATG GTA GGC CCC

DNA restriction and agarose gel electrophoresis. The restriction was conducted in the reaction mixture (10 ц1) containing 4 |ig PCR-product, 20 units of restrictaze and 1 [il of the appropriate buffer. The reaction was terminated by adding 2 ц1 EDTA. The samples were mixed with the dye mixture (0.25% bromophenol blue - 50% glycerin). The genome DNA was separated in 1.5% agarose gel at 5 V/cm in TBE buffer (45 mM Tris-borate, ImM EDTA pH 8).

The GeneDoc, Clone and Oligo 3.1 software were applied to analyze the nucleotide sequences.

Results and discussions

The dominant gene *12* confers resistance in tomato to pathogen races 1 and 2. The locus is mapped to the long arm of chromosome 11 and consists of at least seven genes-homologs belonging to the NBS-LRR resistance gene class [7] (Fig. 1). To find the DNA marker linked with the gene of Fusarium wilt resistance is a rather complicated task because of the lack of the data on the nucleotide sequence of the whole resistance gene cluster *12.* It caused some difficulties for the selecting appropriate primers for the analysis of particular cluster genes due to the possible side amplification of their homologs. The

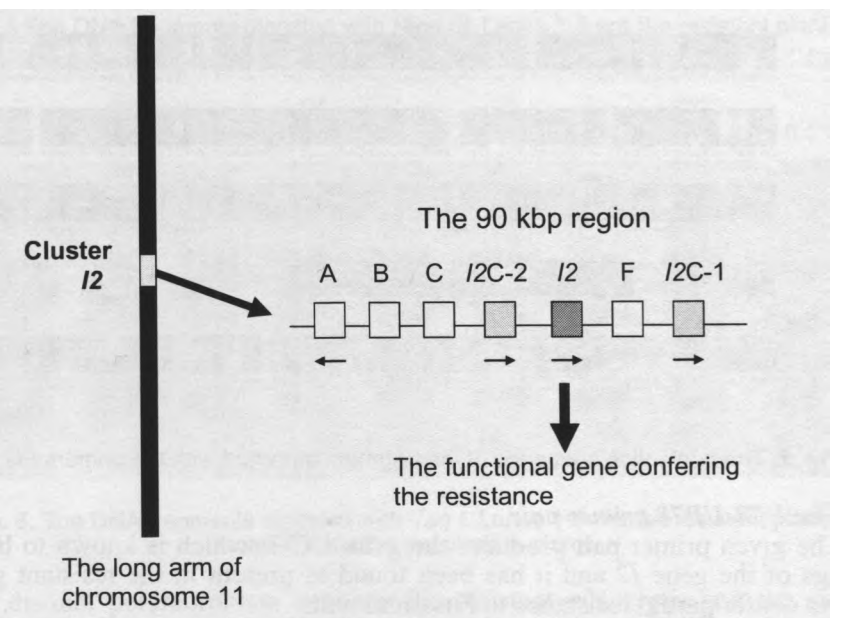

Fig. 1. The **12** resistance gene cluster

other challenge resulted from the lack of information on the nucleotide sequence of DNA region corresponding to the gene cluster *12* in the susceptible genotypes. As a result, the search for the restriction enzymes revealing polymorphism was obscured.

More polymorphic DNA regions were identified via GeneDoc software on the basis of the described cluster *12* homologs sequences. The results obtained were used to design the primers for amplification of the DNA sequences (Fig. 2, see the primers list in Materials and Methods Section). In general, more than 10 primer pairs combined with 20 restrictaze patterns were analyzed. As a result, the primers allowing to analyze the whole sequence of the genes cloned were designed.

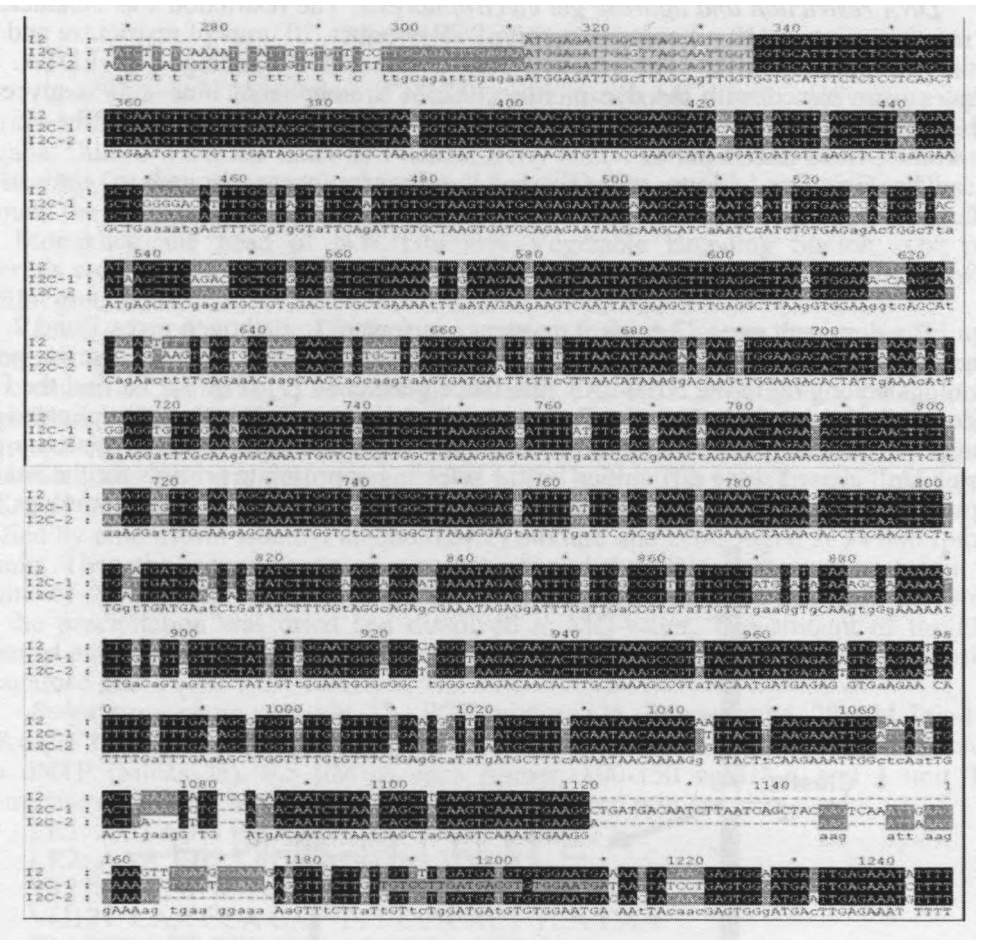

Fig. 2. The nucleotide sequence of the fragment amplified with the primers FG1-LF78

The U78-UP78 primer pair

The given primer pair produces the gene *12C-X,* which is known to be one of the homologs of the gene *12* and it has been found to present in the resistant genotypes as well as to confer partial resistance to Fusarium wilt.

The ten restriction enzymes were tested, and the polymorphism was revealed for the three of them *(Hae III,* Hind III, Taq I - Fig. 3, 4, 5).

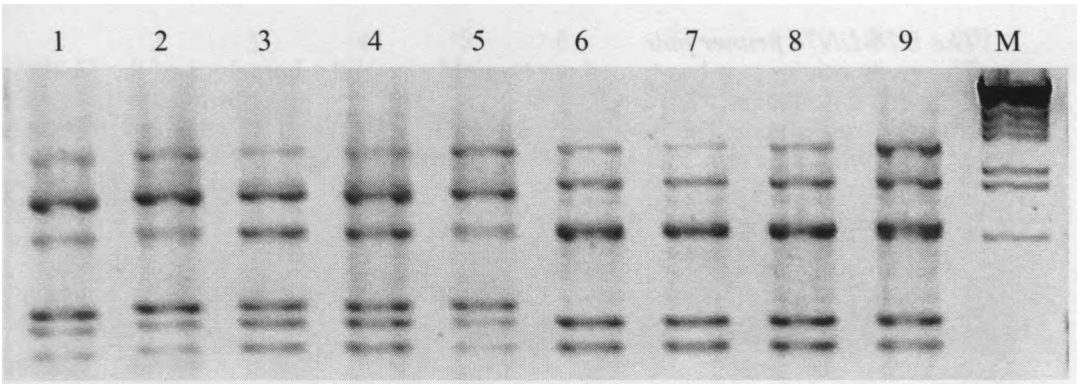

Fig. 3. The DNA fragments digested with *Hae III*. Lanes 1-5 are the resistant plants; lanes 6-9 are the susceptible plants

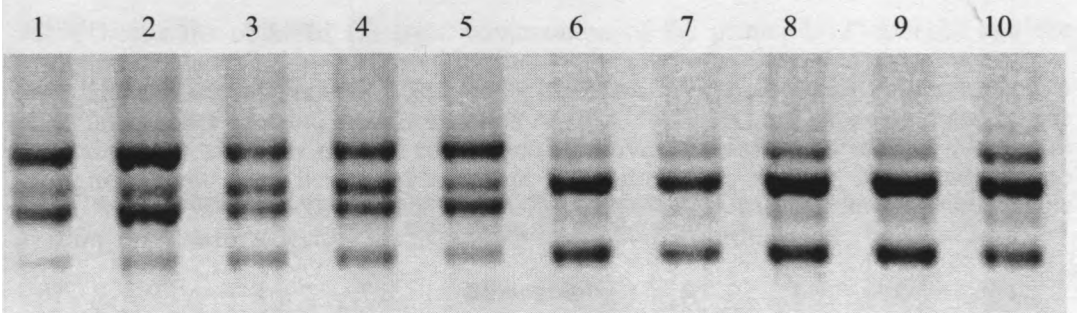

Fig. 4 The DNA fragments digested with *Hind III*. Lanes 1-5 are the resistant plants; lanes 6-9 are the susceptible plants

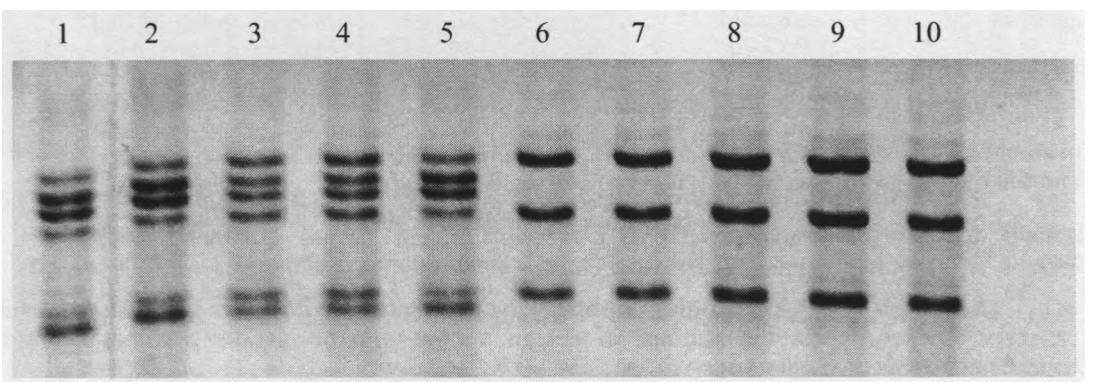

Fig. 5. The DNA fragments digested with *Taq I*. Lanes 1-5 are the resistant plants; lanes 6-9 are the susceptible plants

The distinct polymorphism between the resistant genotypes and the susceptible ones was observed. Nevertheless, the attempt to construct a co-dominant marker detecting homo- and heterozygotes with the primer pair has failed.

The U78-UN78 primer pair

The given primer pair has turned out to yield one of the homologs of the *12,* that is *12*C-l . The latter found in the resistant genotypes and it is responsible for the partial Fusarium wilt resistance. The produced fragment was tested by the ten restriction enzymes, from which the two ones showed polymorphism (Fig. 6, 7).

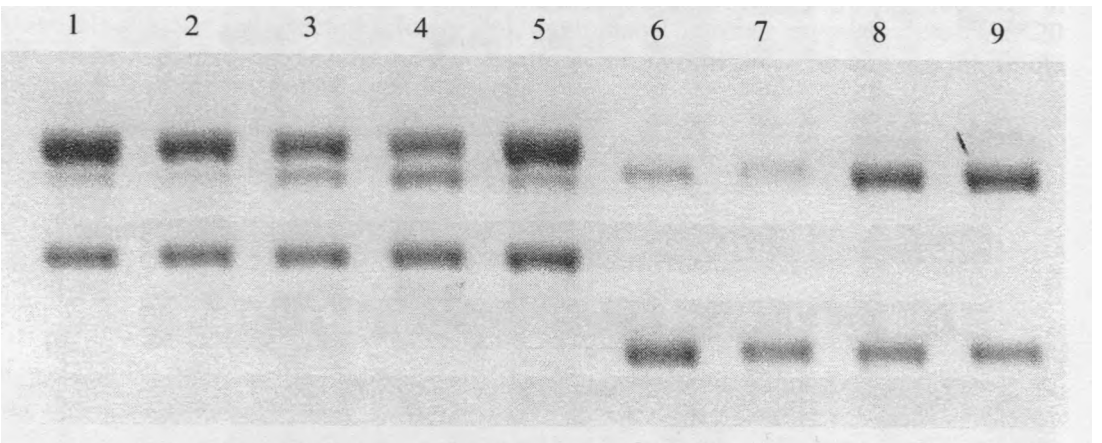

Fig. 6. The DNA fragments digested with Dra /. Lanes **1-5** are the resistant plants; lanes 6-9 are the susceptible plants

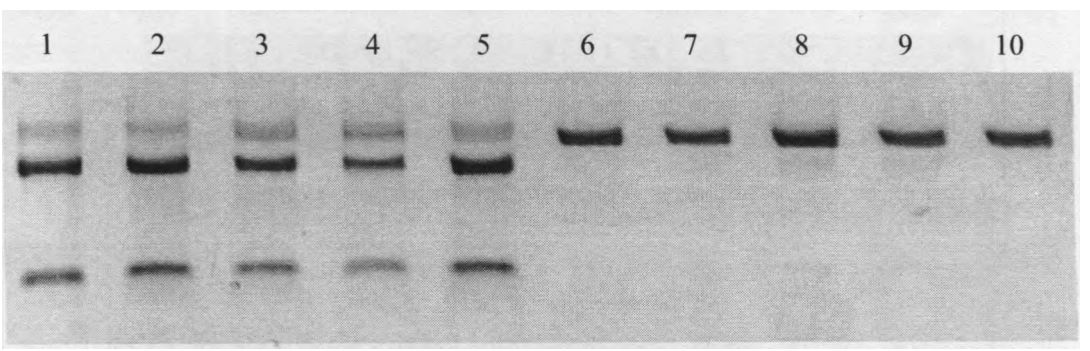

Fig. 7. The DNA fragments digested with *Hha I.* Lanes 1-5 are the resistant plants; I anes 6-9 are the susceptible plants

Although the distinct polymorphism could be noticed between the resistant and susceptible genotypes, the attempt to design a co-dominant marker was not achieve successful as well.

The U127-UN127primer pair

The given primer pair allows to amplify specifically the gene 12 sequence, the latter conferring the resistance to Fusarium wilt. The sequence was digested with the ten different restriction enzymes, some of them revealed polymorphism whereas the one "primer-restrictase" combination has enabled to reveal heterozygotes (Fig. 8).

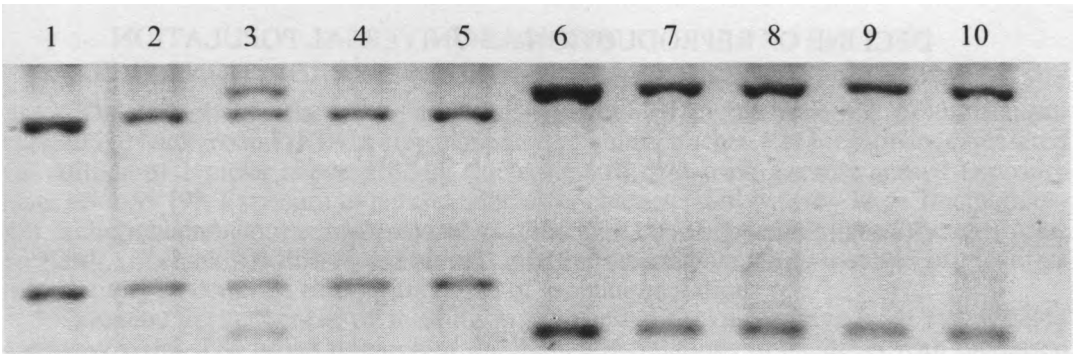

Fig. 8. The DNA fragments digested with *Vha 4641*. Lanes **1-5** are the resistant plants; lanes 6-9 are the susceptible plants. Lane 3 is a heterozygous plant

Conclusion

The results obtained from the combination of the primer U127-UN127 and the restriction enzyme Vha 4641 allowed to classify the marker as the co-dominant one.

The results of tomato genotypes estimation in the artificially infected plots (N.N. Timofeev Vegetable breeding station of RSAU-MTAA) and those obtained by the constructed DNA marker in have coincided. Moreover, the molecular marker allowed to distinguish homo- and heterozygotes from the segregating resistant lines and thus to reduce the breeding process by one selection cycle. So, the marker can be useful in the breeding process for selecting the genotypes resistant to Fusarium wilt.

Bibliography

1. Nelson P. E. // Life cycle and epidemiology of *Fusarium oxysporum* in fungal wilt disease of plant, 1981. Edited by Marshall E. Mace, Alois A. Sell & Carl, and H. Beckman Academic press Inc., 113.

2. Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., Zamir, D., and Fluhr, R. // The *I2C* family from the wilt disease resistance locus *12* belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes, 1997. Plant Cell 9, 521-532.

3. Boumival B. L., Vallejos C. E., Scott J. W. // Genetic analysis of resistances to races 1 and 2 of *Fusarium oxysporum f.sp. lycopersici* from the wild tomato *Solanum pennellii,* 1990. Theoretical-and-Applied-Genetics. 79(5), 641-645.

4. Segal, G., Sarfatti, M., Schaffer, M.A., Ori, N., Zamir, D., and Fluhr, R. // Correlation of genetic and physical structure in the region surrounding the *12* Fusarium oxysporum resistance locus in tomato, 1992. MOI. Gen. Genet. 231, 179-185.

5. Madan Mohan, Suresh Nair, A. Bhagwat, T.G. Krishna, Masahiro Yano, C.R. Bhatia2 and Takuji Sasaki//Genome mapping, molecular markers and marker-assisted selection in crop plants, 1997. *Molecular Breeding* 3, 87-103.

6. Lalitha Sunil Kumar //DNA markers in plant improvement; An overview, 1999. Biotechnology Advances 17, 143-182.

7. Simons, G,, et al. // Dissection of the Fusarium *12* gene clusterin tomato reveals six homologs and one active gene copy, 1998. Plant Cell 10, 1055-1068.

8. Bematzky R., Tanksley S.D.. Toward a saturated linkage mapin tomato based on isozyme and random cDNA sequences. Genetics. 1986, 112: 887-898.

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