Molecular research in the tomato breeding for resistance to biotic stressors

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Abstract. An urgent problem in tomato breeding is the development of universal varieties and hybrids that combine high yields and good fruit quality with group resistance to a number of diseases. The purpose of this study is to develop tomato breeding material resistant to biotic stressors, basing on modern postgenomic technologies using molecular markers. At the initial stage, we tested microsatellite markers from foreign literature sources, the primers used in the study were synthesized by Syntol LLC, samples from the collection of FSBSI Federal Scientific Center for Biological Plant Protection and FSBSI Federal Scientific Rice Centre were studied under the conditions of Krasnodar region. PCR conditions for identifying the target *Ph -3* gene in the tomato plant material used in the breeding process were optimized. Thus, in our study on the testing of microsatellite markers to identify the target *Ph-3* resistance gene in the tomato genetic material, we selected 3 informative microsatellite markers R2M1S, G8-1, R1-3U, which reveal the allelic difference between susceptible and resistant samples.

1 Introduction

Tomato – *Lycopersicon esculentum (Tourn.) Mill* is one of the most important vegetable crops in Russia, its consumption grows every year. It is an important source of vitamins and nutrients. In world and domestic practice, tomato production is significantly damaged by phytopathogenic fungi, of which late blight is one of the most common and harmful. In the southern regions of Russia, late blight is found everywhere, although the intensity of its manifestation in different years is not the same and is mainly determined by weather conditions, resistance of varieties and the racial composition of the pathogen population. Conducted monitoring of economically significant diseases of tomato crops in the conditions of Krasnodar region, 2018–2021 contributed to the spread and development of pathogens that cause alternariosis (*A. alternata*), late blight (P. *infestans*), Fusarium wilt (*Fusarium sp*.), etc. [1]. Field studies conducted in the central soil-climatic zone of Krasnodar region on the territory of experimental plot of FSBSI All-Russian Rice Research Institute, in the period from 2010 to 2021, showed that the most epiphytotic in terms of the

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development of late blight on tomato were 2012, 2013, 2016, 2021. The weather conditions of the growing season of these years were almost ideal for the development of late blight causative agent [2].

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is one of the most harmful diseases, especially in areas with high humidity and low temperatures, and can lead to 100% yield loss both in open and protected ground. Under favorable conditions, P. *infestans* can spread at an alarming rate and a compatible host will be destroyed within 7-10 days. A pathogen population can be affected by several evolutionary mechanisms. In addition to mutation, migration and recombination are often mentioned as the main evolutionary mechanisms that form genetic variability in P. *infestans* populations [3, 4]. The first symptoms of late blight are irregularly shaped, dark brown, water-soaked areas on stems and leaves. A pale green band of tissue usually surrounds the affected area on the leaf. Greyish, moldy fungus growth can be found on the underside of these spots.

The ability to resist tomato late blight lies in the cultivation of resistant varieties. This will make it possible to reduce the environmental burden on agricultural land with less use of chemical plant protection products, as well as reduce crop losses due to pathogenic damage to tomato plants. In addition, the study and conservation of genetic resources of cultivated plants is a key moment in the sustainable development of agriculture in any country. The loss of such resources, and as a result, of genetic diversity, is a harsh reality of our time [5]. There are wild tomato forms and varieties of foreign breeding with an increased level of horizontal resistance. However, there are also such forms among them that either do not ripen in the conditions of Russia, or have no practical value (decorative small-fruited varieties) [6].

For the successful implementation of breeding programs for disease resistance, the search for sources of resistance genes to dominant diseases remains relevant. To date, a number of genes with vertical resistance have been found in tomato, respectively, three specific resistance genes for each late blight race (*Ph 1, Ph 2* and *Ph 3*). The *Ph-1* gene (chromosome 7) is a dominant gene that provides resistance to the T0 race and several isolates of the T1 race. The *Ph-2* gene (chromosome 10) is an incompletely dominant gene, provides partial resistance to several isolates of the T1 race, slows down but does not stop the progression of the disease. *Ph*-3 gene (chromosome 9) of resistance to late blight (P. *infestans*), provides incomplete dominant resistance to a wide range of P. *infestans* tomato isolates, including those that overcome *Ph-1* and *Ph-2* genes [7, 8, 9, 10]. It was this gene that was used and introgressed by us into domestic tomato varieties to increase their immunity.

The use of new biotechnological tools opens up prospects for the accelerated development of valuable genotypes in a short time, which is technically unattainable by classical breeding methods. One of the new biotechnological paradigms that could have the greatest impact are molecular markers. They allow not only to control the presence of target genes in the hybrid breeding material, but also to track their allelic state. Molecular markers of resistance alleles significantly improve the selection of resistant plants [3].

Due to the fact that the need to develop genetic sources of agricultural plants resistant to biotic stressors, namely late blight, is increasing, the purpose of this work was to select informative markers available from foreign sources that provide a clear control of the inheritance of target genes/loci for resistance to P. *infestans*. The selection of markers suitable for assessing the DNA polymorphism of tomato varieties that differ in resistance to phytophthora was carried out on material from the collection of FSBSI “Federal Scientific Rice Centre” and FSBSI “Federal Scientific Center for Biological Plant Protection”, studied under the conditions of Krasnodar region.

An important and urgent problem in plant breeding is development of breeding material with desired properties. Currently, breeders are striving to increase the competitiveness of

their products in a situation of strong competition in the seed market, including vegetable crops (tomatoes). The application and integration of new technologies for molecular genetic evaluation of the source material in the breeding process will significantly reduce the time for developing genotypes with desired properties.

2 Materials and methods

Plant material

In this study, we used 5 tomato genotypes from the collection of FSBSI "Federal Scientific Rice Centre" and FSBSI "Federal Scientific Center for Biological Plant Protection", which have increased immunity to Fusarium, Tobacco Mosaic Virus, Cladosporiosis, Verticillium, these include line-4 and line-1; to the Tomato spotted wilt virus, this is line-13; and have positive morphometric characteristics (the color of the unripe fruit is dark green, the color of the mature fruit is red, the weight of fruit is 80 grams (in an artificial climate chamber), the taste of the fruit is good); the value of line-4 and line-1: early ripeness, high yield, uniformity of fruits.

DNA extraction

For molecular genetic studies, DNA of the analyzed samples is isolated from a freshly cut part of the leaf blade of tomato plants at the stage of 4-5 true leaves according to the Murray and Thompson method, using cetyltrimethylammonium bromide as the main lysis buffer with modifications (CTAB) [11]. DNA extraction is carried out with a buffer of the following composition: 1M Tris-HCl (pH 7.5), 5M NaCl, 0.5M EDTA (pH 8.0), 10% SDS. A part of the leaf (2–3 cm) was triturated in 500 µl of extraction buffer in a 1.5 ml plastic tube.

Samples are incubated at 60°C for 2 hours. Then cooled to room temperature. The supernatant is separated by centrifugation at 12000 rpm. 500 µl of isopropanol is added to the upper phase transferred into a clean test tube and left for 30 minutes after mixing. After that, the samples are centrifuged for 5 minutes at 12000 rpm. The precipitate obtained is washed with 300 µl of 70% ethanol, dried and dissolved in 50 µl of 0.1*TE. After DNA extraction from tomato plant material, a Master Mix solution is added to it, which consists of: PCR buffer 10x (Mg), MgCl2 (25 mM), dNTPs mixture (25 mM), primers, Taq polymerase (5U / µl), H_2O (for injection) at the rate of 25 µl per 1 sample. 2-3 drops of vaseline oil were added to the test tubes over the reaction mixture to prevent evaporation during amplification. When carrying out the amplification reaction, microsatellite molecular markers with specific primers associated with the locus responsible for resistance to tomato fungal disease are used. If the marker analysis method makes it possible to identify both alleles, they speak of a codominant type of marker inheritance (monolocus), if only one allele is detected, it is a dominant type (multilocus) [12]. At the same time, the accuracy of estimates using codominant markers is higher, because their heterozygous state differs from homozygous, in contrast to dominant markers. The efficiency of breeding for immunity depends on the quality of plant resistance donors; for this purpose, a DNA analysis of tomato genetic material was carried out for the presence of genes / loci of interest in the studied samples. In this study, we tested markers linked to the gene (*Ph-3*) of resistance to late blight, taken from the literature, the data are presented in Table 1. These primers make it possible to isolate tomato plants with genes that control vertical resistance to late blight: *Ph-3* on chromosome 9.

Table 1. Description of the markers used in the study.

Marker name	Forward primer (5/ –3/)	Reverse primer (5/ –3/)	Marker type	Literary source
UF-Ph3-5ᶻ	TGGAACAATTTTCACCACCCA	TGACAAAGGACAAATGCATGG	SCAR	(Reza Shekastebandet al. 2015) [13]
Ph3-SCAR	CTACTCGTGCAAGAAGGTAC	TCCACATCACCTGCCAGTTG	SCAR	(Jungsu Jung et al. 2015) [14]
R2M1S	GGAAATCCTCCGCCTTACTT	CGAGTTGCAACCTCTAGACTCA	Co-dominant SCAR	(Chunzhi Zhang et al. 2014) [15]
TG591S	GCGAGACATAGACCAATC	AACTGGCAGGTGATGTGG	SNP	
G8-1	CGCCGTTTCGTGGCATTT	AGCGTGGTGATGGTGTTT	SNP	
G7-5	TGCCTCTGTGAAGATGGT	AAACTGTCGCAGGGTATT	SNP	
R1-3U	AAAAGTATTCAGAGGGGTAA	ATTGCAGATCCATTTCAGT	Co-dominant SCAR	

Amplification is carried out in a DNA-amplifier, under the following conditions:

For the UF-Ph3-5ᶻ SCAR marker (SCAR is a nucleotide sequence characterizing the amplified region): at the first stage, denaturation takes place for 30 seconds at 98 ℃, then at the second stage, 42 cycles are performed according to the following protocol: denaturation - 5 seconds at 98 °C; primer annealing - 5 sec at 56 °C; extension - 20 sec at 72 °C. The final stage includes one cycle of extension at 72 °C for 1 minute. For the Ph3-SCAR marker: at the first stage, denaturation takes place within 15 minutes at 95 ℃, then at the second stage 40 cycles are carried out according to the following protocol: denaturation - 30 sec at 95 °C; primer annealing - 15 sec at 57 °C; extension - 30 sec at 72 °C. The final stage includes one cycle of extension at 72°C for 5 minutes. For the R2M1S -SCAR marker: the first step is denaturation for 4 minutes at 94 ℃, then at the second stage 35 cycles are carried out according to the following protocol: denaturation - 30 sec at 94 °C; primer annealing - 60 sec at 55°C; extension - 60 sec at 72 °C. The final stage includes one cycle of extension at 72°C for 5 minutes. For TG 591S-SNP markers; G8-1-SNP; G7-5-SNP; R1-3U-SCAR: at the first stage, denaturation takes place for 4 minutes at 94 ℃, then at the second stage 35 cycles are carried out according to the following protocol: denaturation - 30 seconds at 94 °C; primer annealing - 60 sec at 50°C; extension - 60 sec at 72 °C. The final stage includes one cycle of extension at 72 °C for 5 minutes.

Amplification products were separated by electrophoresis:

1) in agarose gel, the optimal concentration of agarose (2%) was selected, at which PCR products are clearly visualized. Its use significantly reduces the time; 2) in 8% polyacrylamide gel (PAAG) at a voltage of 3.9-4.5 V/cm [16].

To visualize the results of the electrophoretic separation of PCR products, a plate of agarose or polyacrylamide gel was placed in the GelDocXR+ device, and using a special program, according to the instructions, the gel under study was photographed in ultraviolet light and the data obtained were analyzed. To determine the length of the amplified fragments, a molecular weight marker was used: M -100 (Synthol).

3 Results and discussion

The use of DNA marking technologies in the breeding paradigm significantly accelerates development of modern genotypes with desired properties, their introduction to the market, increasing competitiveness and reducing import dependence on foreign companies. At the first stage of the breeding program for development of tomato genotypes with increased immunity to P. *infestans*, we selected variety samples contrasting in terms of resistance to late blight, namely, the differentiator variety Ottawa 30 (for the tomato late blight race T1) [1,17] and susceptible line 4, as well as samples with positive morphological and biological characteristics and resistance to other dominant diseases in the south of Russia (fusarium and tomato spotted wilt virus). Their DNA polymorphism was studied using microsatellite molecular markers. The results are presented in Figures 1-4.

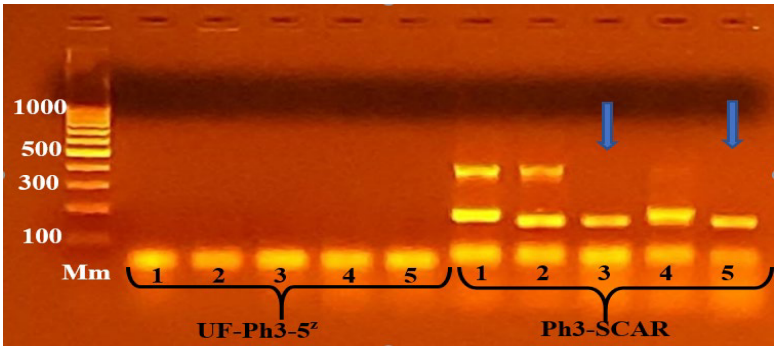

Fig. 1. Visualization of PCR-products in 2% agarose gel.

Note: Mm – molecular weight marker M-100 (supplier – Syntol, Russia); 1-susceptible form (line-4); 2 – sample (line-1) with positive morphometric characteristics; 3 mutant form - (F2 Mo 628×L peruvianum var humifusum); 4 - stable heterozygote for tomato spotted wilt virus with positive morphometric characteristics (line-13); 5 - Ottawa 30 (resistant line).

Figure 1 shows that for the UF-Ph3-5ᶻ locus, all the studied samples did not show an allele difference (polymorphism) and, therefore, the marker is not effective and not suitable for use in identifying donor alleles of the target *Ph-3* gene. Analyzing the data on the Ph3-SCAR locus, it was found that the sample No. 3 (mutant form) has an identical DNA profile with a resistant sample No. 5.

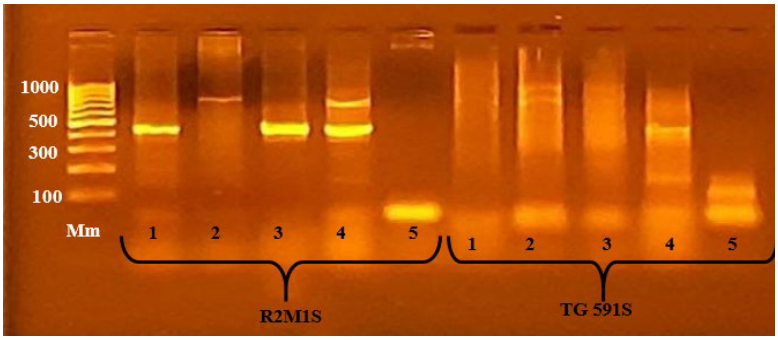

Fig. 2. Visualization of PCR-products in 2% agarose gel.

Note: Mm – molecular weight marker M-100 (supplier – Syntol, Russia); 1- susceptible form (line-4); 2 – sample (line-1) with positive morphometric characteristics; 3 - mutant form (F2 Mo 628×L peruvianum var humifusum); 4 – stable heterozygote for tomato spotted wilt virus with positive morphometric characteristics (line-13); 5 – Ottawa 30 (resistant line).

Figure 2 shows that polymorphism between the analyzed samples for the TG 591S locus was not detected and this marker is not suitable for visualizing donor alleles of the target gene. For the R2M1S locus, in all analyzed tomato samples, the DNA profile in agarose gel differs from resistant control (Ottawa 30), and we additionally visualized this marker in an 8% polyacrylamide gel (Figure 3).

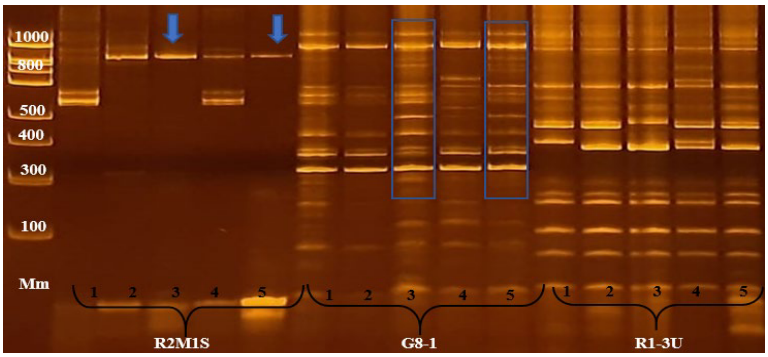

Fig. 3. Visualization of PCR-products in 8% polyacrylamide gel.

Note: Mm – molecular weight marker M-100 (supplier – Syntol, Russia); 1- susceptible form (line-4); 2 – sample (line-1) with positive morphometric characteristics; 3 - mutant form (F2 Mo 628×L peruvianum var humifusum); 4 – stable heterozygote tomato spotted wilt virus with positive morphometric characteristics (line-13); 5 – Ottawa 30 (resistant line).

Figure 3 shows the results of visualization of PCR products in polyacrylamide gel. According to this R2M1S microsatellite locus, it can be said that sample No. 3 has a similar DNA profile with sample No. 5, as well as for the Ph3-SCAR locus. Looking at the G8-1 locus, it can be seen that sample No.3 also has an identical DNA profile with stable sample No. 5. Analyzing the data on the R1-3U locus, it can be seen that sample No. 2 has a DNA profile similar to that of resistant sample No. 5.

Molecular markers R2M1S, G8-1, R1-3U will be used by us to screen F_2 progeny. If the data on the phenotype coincide with the genotype analysis, then they can be recommended for the breeding process to identify the target *Ph-3* gene and its allelic state, which will significantly accelerate the scheme of the breeding process in this direction.

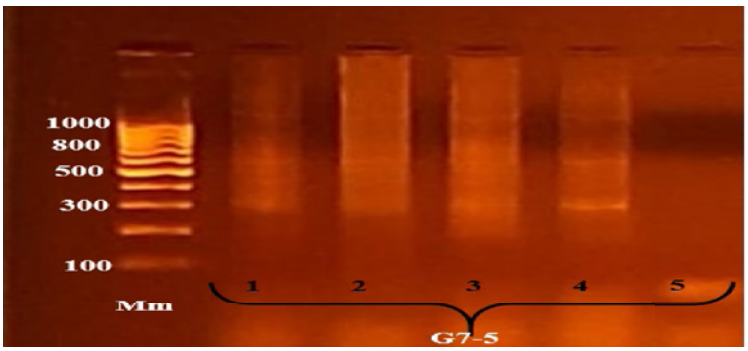

Fig. 4. Visualization of PCR-products in 2% agarose gel.

Note: Mm – molecular weight marker M-100 (supplier – Syntol, Russia); 1- susceptible form (line-4); 2 – sample (line-1) with positive morphometric characteristics; 3 - mutant form (F2 Mo 628×L peruvianum var humifusum); 4 – stable heterozygote tomato spotted wilt virus with positive morphometric characteristics (line-13); 5 – Ottawa 30 (resistant line).

Figure 4 demonstrates that no allelic difference between resistant and susceptible samples was found at the G7-5 locus; this marker is also not suitable for visualizing donor alleles of the target gene.

4 Conclusion

Thus, basing on the results obtained, for the studied tomato plant genotypes, sample No. 3 showed the presence of the target gene as well as a donor of the specific late blight resistance gene Ottawa 30 for the marker R2M1S, G8-1. Therefore, sample No. 3 is homozygous for the target *Ph-3* gene and can be recommended for use in breeding. At the next stage of our study, we will screen the breeding material of tomato plants used by the Department of Vegetable and Potato Growing of FSBSI "Federal Scientific Rice Centre" in the breeding program.

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