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FEATURES OF THE PRIMARY STRUCTURE OF THE *Ph-3* GENE, REVEALED BY DEVELOPMENT OF A NEW GENE-BASED MARKER OF LATE BLIGHT RESISTANCE IN TOMATO

V.V. MARTYNOV[1] ✉, E.G. KOZAR'[2], I.A. ENGALYCHEVA[2]

[1]*All-Russian Research Institute of Agricultural Biotechnology,* 42, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail martynov.vik@gmail.com (✉ corresponding author);
[2]*Federal Research Center for Vegetable Growing*, 14, ul. Selektsionnaya, pos. VNIISSOK, Odintsovskii district, 143072 Russia, e-mail kozar_eg@mail.ru, engirina1980@mail.ru
ORCID:
Martynov V.V. orcid.org/0000-0003-1784-3429
Kozar' E.G. orcid.org/0000-0002-1319-5631
I.A. Engalycheva orcid.org/0000-0003-4843-111X
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Abstract

Late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is one of the most harmful diseases of tomatoes. Late blight control remains challenging due to the high genetic variability and complex racial composition of *P. infestans*. Therefore, the most promising method of combating late blight is the breeding of resistant varieties of tomato. When creating resistant varieties, the introgression of resistance genes from wild-growing related species is widely used. In particular, several late blight resistance genes identified in the wild tomato species *Solanum pimpinellifolium* have been introgressed into tomato cultivars. Among these genes, the *Ph-3* gene is considered to be the strongest late blight resistance gene, as it provides resistance to a variety of *P. infestans* isolates. Therefore, considerable efforts of scientific groups around the world are directed to the study of this gene in order to include it in breeding programs and introduce it into new commercial varieties and lines of tomato. To date, DNA markers associated with this gene are known. However, homologues of this gene were found in the tomato genome, which do not have functional activity. Analysis of the multiple alignment of the nucleotide sequences of the *Ph-3* gene and its homologues showed that the primers used in the known markers for amplification of this gene are in the conservative regions of these sequences, and it is impossible to specifically amplify the *Ph-3* gene with them. Therefore, the aim of this work was to design a new highly specific marker of the *Ph-3* gene and compare it with already known markers by analyzing the collection of tomato varieties of the Federal Scientific Center for Vegetable Growing for the presence of known and new markers and assessing the linkage of these markers with resistance to late blight disease in the studied varieties. To this end specific primers were designed (5′-AATATTGAAAATAGCTGCACTGA-3′/5′-CGAGATTTGGAGGGAATGTAA-3′) that discern the *Ph-3* gene from its homologues and amplify a 412 bp gene fragment (the Ph3-412 marker). Using these primers, 24 tomato (*Solanum lycopersicum* L.) varieties bred at the Federal Scientific and Technical Center and tested for late blight field resistance (Federal Scientific and Technical Center, Moscow Province, 2021) were analyzed. Also, these varieties were analyzed with known marker NC-LB-9-6678. To determine the nucleotide sequence of the new marker, we cloned the amplified product obtained from the studied varieties into pAL-TA vector and sequenced the resulting clones. In addition, we cloned and sequenced 601 and 907 bp fragments obtained with a known marker. We compared the nucleotide sequences of all three fragments with the sequences of the prototype gene and its known homologues. As a result, we confirmed that the fragment amplified using primers designed by us belongs to the *Ph-3* gene, while the 601 bp fragment obtained with the known primers corresponds to the *SlRGA4* homologue, and the 907 bp fragment obtained with the same primers is homologous to the *Ph-3* gene but it contains an insertion of the LTR retrotransposon of the Ty1-*copy* family with a size of 306 bp. Thus, the gene containing such insertion is most likely inactive. We also showed that in all analyzed varieties, in which the *Ph-3* gene was found, this gene contains the above-mentioned insertion. The presence of such insertion can lead to a loss of functional activity; this must be taken into account when marking the *Ph-3* gene. For the breeding programs it is necessary to identify plants in which the *Ph-3* gene does not have this retrotransposon insertion.

Keywords: tomatoes, late blight disease, *Ph-3* gene, DNA markers, resistance genes

Late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is one of the most harmful tomato diseases that can destroy up to 100% of the crop [1]. Late blight control remains challenging due to the high genetic variability and complex racial composition of *P. infestans*. The use of fungicides, in particular metalaxyl, is not effective enough, since pathogen races quickly mutate and acquire resistance to this drug [2-6]. In addition, fungicide treatment is expensive, and fungicides themselves are harmful to the environment and dangerous to human health. The most promising method of combating late blight is the breeding of resistant varieties of tomato.

When creating resistant varieties, the introgression of genetic material from wild-growing related species is widely used in order to pyramid their resistance genes [7-9]. In particular, several late blight resistance genes identified in the wild tomato species Solanum pimpinellifolium have been introgressed into cultivars [10, 11]. These are the *Ph-1*, *Ph-2* and *Ph-3* genes. The *Ph-1* gene, mapped on chromosome 7, confers resistance to the T0 race of *P. infestans* [12]. The *Ph-2* gene, originally identified in *S. pimpinellifolium* West Virginia 700 (WV700) in chromosome 10, provides resistance to the T0 race and partial resistance to the T1 race [13]. However, the resistance determined by these genes is quickly overcome by new races of *P. infestans*. The *Ph-3* gene was identified in the L3708 sample of S. pimpinellifolium and mapped to the long arm of the chromosome 9. It provides resistance to many races of *P. infestans* that overcome the resistance conferred by the *Ph-1* and *Ph-2* genes [14]. *Ph-2* and *Ph-3* have also been shown to act synergistically and together confer resistance to a broader range of pathogen isolates than either gene alone [15].

Currently, the *Ph-3* gene is considered to be the strongest gene for resistance to tomato late blight. Considerable efforts have been directed to its study in order to include it in breeding programs and introduce it into new commercial varieties and lines [16]. The *Ph-3* gene was cloned and characterized at the molecular level from the L3708 specimen of *S. pimpinellifolium*. *Ph-3* has been found to encode a protein containing a supercoil domain, a nucleotide-binding domain, and leucine-rich repeats (CC-NBS-LRR). When transgenic, it can confer late blight resistance in susceptible tomato varieties [17]. Four structural homologues of this gene, the *SlRGA1*, *SlRGA2*, *SlRGA3*, and *SlRGA4*, were found in the locus corresponding to the *Ph-3* locus of *S. pimpinellifolium* in the genome of the tomato cv. Heinz1706.

Efforts are also being made to mark the *Ph-3* gene for more efficient transfer to material of interest by marker assistant selection (MAS) and pyramiding with other late blight resistance genes. To date, several DNA markers are known to be somehow associated with this gene, including three SCAR (sequence characterized amplified region) markers [18-20] and one CAPS (cleaved amplified polymorphic sequences) marker [21]. However, these markers have a number of disadvantages.

In particular, with the help of SCAR markers described by Y. Park et al. [18], homologues of the *Ph-3* gene can be distinguished, but not the gene itself. In addition, the analysis is proposed to be carried out using a set of three pairs of primers, which increases labor and time costs and complicates the interpretation of the results. The marker described by H.T.H. Truong et al. [19], was derived from a RAPD marker, and its relationship to the *Ph-3* gene sequence is unknown. Multiple alignment analysis of the nucleotide sequences of the *Ph-3* gene and its known homologues showed that the primers used by D.R. Panthee et al. [20] for amplification of this gene, are located in the conserved regions of the mentioned sequences and it is not possible to specifically amplify the gene with their help. CAPS marker described by Y.-Y. Wang et al. [21], involves the use of restriction

endonucleases and the separation of restriction products in a polyacrylamide gel, which makes this analysis relatively expensive and time consuming. In addition, in the case of CAPS markers, there may be problems with the reproducibility of results, since the restriction efficiency is affected by the activity of the enzyme and the amount of DNA, and these parameters are difficult to accurately control. In addition, the validation of all these markers was carried out on segregating populations obtained by crossing susceptible and resistant parental forms.

In the present work, it was shown for the first time that in the varieties of tomato of domestic selection in the presence of the *Ph-3* gene, its other homologues are absent. It was also established for the first time that a retrotransposon insertion is present in the *Ph-3* gene sequence, which can lead to the loss of the genome's functional activity.

Our goal was to design an easy-to-use, highly specific DNA marker for the *Ph-3* gene to be used to distinguish *Ph-3* from its structural homologues. In addition, we aimed at validation of this marker in comparison with already known markers based on the analysis of the collection of domestic varieties and lines of tomato and the assessment of the relationship of markers with field resistance to late blight.

Materials and methods. The study was performed on 24 samples of tomato (*Solanum lycopersicum* L.) bred by the Federal Scientific Center for Vegetable Growing (FNTSO) and included in the State Register of Breeding Achievements approved for use in the Russian Federation, These are Ottawa 30 (late blight resistance standard), Talalikhin (susceptibility standard to late blight), Fitilek, Primorets, Grot, Charovnitsa, Lotus, Rosinka, Pos'yet, Toptyzhka, Odyssei, Patrocl, Blagodatny, Viking, Dubok, Revansh, Talisman, Monakh, Perst, Kameya, Severyanka and Voskhod VNIISSOKa, lines l-DVot30- 2/19 and l-Ft5/20. Thirty plants of each variety and line were grown under laboratory conditions up to 4 weeks of age at 23-25 °C, air humidity of 70-80% and 16 hours of artificial lighting (from 7.00 to 23.00), after which they were transplanted into open ground.

The experiments were carried out in 2021 on the experimental field of the Federal Scientific Center for Vegetable Growing (Moscow Province, Odintsovo District) against a provocative infectious background (an isolated area with a monoculture of tomato). Seedlings were planted at 4-5 true leaves in the first ten days of June using a two-line planting according to the scheme 70×40 cm for determinant varieties, 70×35 cm for standard varieties. Agrotechnics for growing seedlings was standard for tomato culture.

For the phenotypic assessment of tomato resistance to *P. infestans*, 10 plants of each accession were planted in 3 replicates according to the scheme of randomized blocks so that each accession had the same chances of infection. To control the evenness of the infectious background and the dynamics of the development of the disease, the susceptible variety Talalikhin and the resistant variety Ottawa 30 were used, which were planted through five studied samples. Accounting for late blight lesions was carried out in dynamics every 7 days, starting from the appearance of the first symptoms (III decade of July). Plant damage was assessed visually by characteristic symptoms according to a modified ten-point scale where 0 means no symptoms; 0.1 means 1-5% affected leaf area, small lesions (<2 mm), no stem lesions; 0.5 means 6-10% affected leaf area, no damage to the stems; 1 means 11-20% affected leaf area, no damage to the stems; 1.5 means 21-30% affected leaf area, confluent leaf lesions or tiny watery stem lesions; 2 means 31-40% affected leaf area, expanding along the edges of the leaf lesion or several small stem lesions (< 5 mm); 2.5 means 41-50% affected leaf area, stem lesions (< 30 mm); 3 means 51-60% affected leaf area, drying damage to the leaves or damage to the stem with the expansion of the edges, 20% affected fruits; 3.5 means

61-70% affected leaf area, drying damage to the leaves and damage to the stem with the expansion of the edges, 40% affected fruits; 4 means 71-100% affected leaves, stems and fruits. The resistance of each sample was assessed by the lesion index (I, average score). According to the totality of all assessments, the samples were differentiated into resistance groups, R for resistant (I = 0), RS for relatively stable (0 < I ≤ 1), MS for moderately susceptible (1 < I ≤ 2), S for susceptible (2 < I ≤ 3), HS are highly susceptible (I > 3).

Total DNA was extracted from young leaves of 2-week-old plants using the Sorb-GMO-B reagent kit (Synthol, Russia) according to the manufacturer's protocol. For each variety, DNA was isolated from all 8 plants, after which the DNA preparations were combined into one common sample.

The design of primers for specific amplification of the *Ph-3* gene was based on multiple alignment of the nucleotide sequence of the *Ph-3* gene (GenBank no. KJ563933) and its structural homologues *SlRGA1*, *SlRGA2*, *SlRGA3*, and *SlRGA4*, the nucleotide sequences of which were taken from the nucleotide sequence of the tomato chromosome 9 registered in the GenBank NCBI database (GenBank no. EF647605.1). Forward (5′-AATATTGAAAATAGCTGCACTGA-3′) and reverse (5′-CGAGATTTGGAGGGAATGTAA-3′) primers were designed in which the sequences of the 3′-ends were strictly specific for the *Ph-3* gene and differed from the sequences of the gene homologues in this position. These primers were located in the LRR domain of the *Ph-3* gene, and the expected amplicon size was 412 bp. In addition, for comparative analysis, primers of the marker NC-LB-9-6678 5′-CCTTAATGCAATAGGCAAAT-3′ and 5 -ATTTGAATGTTCTG-GATTGG-3′ [11] were used the sequences of which were absolutely conserved for the *Ph-3* gene and its homologs.

The amplification program was 3 min at 94 °C; 30 s at 94 °C, 30 s at 60 °C, 1 min at 72°C (35 cycles); 5 min at 72 °C (final synthesis). The volume of the reaction mixture was 25 μl. For one reaction, 50 ng of total DNA was taken. For amplification, a GeneAmp PCR System 2700 device (Applied Biosystems, Inc., USA) was used. Electrophoretic separation of amplification products was carried out in 1% agarose gel with 1× TAE buffer. Amplification conditions with primers NC-LB-9-6678 were as described by D.R. Panthee et al. [20], 3 min at 92 °C; 30 s at 92 °C, 1 min at 52 °C, 30 s at 72 °C (35 cycles); 8 min at 72 °C (final alongation).

For nucleotide sequencing, the obtained amplicons were cloned into the pAL-TA vector (Evrogen, Russia), which was used to transform competent cells of *Escherichia coli* DH5α, and sequenced by the Sanger method using the Big Dye Terminator v.3.1 reagent kit (Applied Biosystems, Inc., USA; an ABI PRIZM 3730 automatic sequencer, Applied Biosystems, Inc., USA) according to the manufacturer's instructions.

Multiple alignment of nucleotide sequences was performed using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clust-alo/) followed by analysis of the alignment results with the GeneDoc 2.7 program (https://genedoc.software.informer.com/2.7/). The TREECON program [22] was used to construct the dendrogram. Derived amino acid sequences were obtained using the EditSeq program (https://macdownload.informer.com/editseq/download/). The BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for homologues of the obtained sequences in the NCBI database.

Results. During PCR amplification of total DNA preparations isolated from 24 tomato samples with primers specific for the *Ph-3* gene, the Ph3-412 marker we created was found in all analyzed samples, except for three (l-Ft5-19, Viking and Revansh) (Fig. 1, A). According to the results of analysis with primers to the marker NC-LB-9-6678, three of these samples had a marker, which in the

work of H.L. Merk et al. [11] was associated with resistance to late blight of tomato (600 bp), and the rest were a marker associated with susceptibility (900 bp) (see Fig. 1, B). In other words, the Ph3-412 marker was absent in varieties in which the previously known marker associated with resistance was found, and was present in varieties in which a 900 bp fragment associated with susceptibility to late blight was detected. That is, an analysis with a new and previously known marker gave diametrically opposite results.

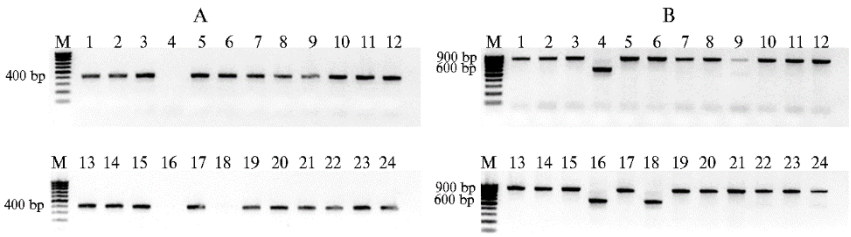

Fig. 1. Electrophoregram of PCR amplification products of total DNA of tomato (*Solanum lycopersicum* L.) samples with primers of markers Ph3-412 (A) and NC-LB-9-6678 (B), specific for the late blight resistance gene *Ph-3*: M — molecular weight marker, 1 — Ottawa 30, 2 — l-DVot30-2/19, 3 — Fitilek, 4 — l-Ft5/20, 5 — Primorets, 6 — Grot, 7 — Charovnitsa, 8 — Lotus, 9 — Talalikhin, 10 — Rosinka, 11 — Pos'yet, 12 — Toptyzhka, 13 — Odyssei, 14 — Patrocl, 15 — Blagodatny, 16 — Viking, 17 — Dubok, 18 — Revansh, 19 — Talisman, 20 — Monakh, 21 — Perst, 22 — Kameya, 23 — Severyanka, 24 — Voskhod VNIISSOKa

Comparison of the results of molecular analysis using DNA markers of the late blight resistance gene *Ph-3* with the data of phenotypic assessment of field resistance to late blight in tomato (*Solanum lycopersicum* L.) samples

Variety	Resistance group by phenotype	Marker		
		Ph3-412	NC-LB-9-6678 600 bp	NC-LB-9-6678 900 bp
Ottawa 30 (resilience standard)	SR	+	–	+
Fitilek	MS	+	–	+
Lotus	MS	+	–	+
Patrocl	MS	+	–	+
Primorets	MS	+	–	+
Blagodatny	MS	+	–	+
Talisman	MS	+	–	+
Monakh	MS	+	–	+
L-DWot30-2/19	MS	+	–	+
Revansh	MS	–	+	–
Viking	MS	–	+	–
l-Ft5/20	S	–	+	–
Talalikhin (susceptibility standard)	S	+	–	+
Rosinka	S	+	–	+
Charovnitsa	S	+	–	+
Toptyzhka	S	+	–	+
Dubok	S	+	–	+
Perst	S	+	–	+
Kameya	S	+	–	+
Voskhod VNIISSOKa	S	+	–	+
Pos'yet	HS	+	–	+
Grot	HS	+	–	+
Odysseus	HS	+	–	+
Severyanka	HS	+	–	+

N o t e. SR — semiresistant, MS — medium susceptible, S — susceptible, HS — higly susceptible; «+» — present, «–» — absent.

The weather conditions of 2021 were characterized by a hot and dry growing season: precipitation for the entire period was 29.4 mm less than the long-term average, the air temperature was 2.5 °C above the climatic norm. Accounting for the damage of varieties-differentiators by the oomycete *P. infestans* showed the presence of the T1 race, which is characterized by high aggressiveness and virulence in open ground conditions in 2021.

When comparing the results of molecular analysis with the data of phenotypic assessment of field resistance to late blight, it was found that the Ph3-412 marker was present in the Ottawa 30 variety resistance standard, and only a 900 bp fragment was noted for the NC-LB-9-6678 marker, while a 600 bp fragment associated with resistance was absent (Table). Of the nine moderately susceptible samples, the Ph3-412 marker was found in seven samples, the 600 bp resistance marker NC-LB-9-6678 in two samples. Among susceptible samples, the Ph3-412 marker was detected in 13 samples, and a known resistance marker was detected in one sample. Consequently, none of the markers showed an unambiguous association with field stability.

To find out what may cause such ambiguity, we cloned and sequenced 412 bp PCR products obtained with primers Ph3-412, 600 bp and 900 bp PCR products obtained with primers NC-LB-9-6678. The obtained nucleotide sequences were compared with the sequence of the Ph-3 prototype gene and its structural homologues SlRGA1, SlRGA2, SlRGA3, and SlRGA4. The comparison results are presented in the form of dendrograms (Fig. 2).

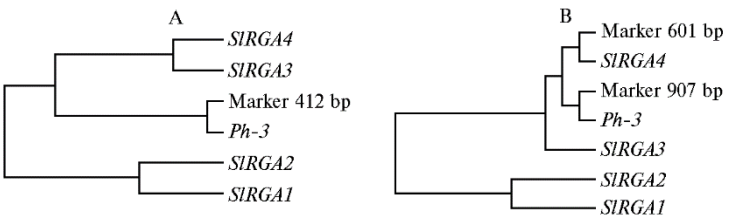

Fig. 2. Dendrograms based on comparing the nucleotide sequences of the tomato (*Solanum lycopersicum* L.) late blight resistance gene *Ph-3* and its homologues *SlRGA1*, *SlRGA2*, *SlRGA3*, *SlRGA4* with the nucleotide sequence of the 412 bp PCR product generated with the primers of the Ph3-412 marker (A)**, and nucleotide sequences of 601 bp and 907 bp PCR products generated with the primers of the NC-LB-9-6678 marker** (B)**.**

The fragment amplified with the Ph3-412 primers clustered together with the *Ph-3* prototype gene, and its nucleotide sequence was 99.3% homologous to the *Ph-3* sequence (see Fig. 2, A). That is, we can confidently state that the primers we created specifically amplify the *Ph-3* gene and not its homologues. The exact sizes of the fragments amplified with primers NC-LB-9-6678 were 601 bp. and 907 bp. It turned out that the 601 bp fragment belongs to the *SlRGA4* homologue, on the dendrogram, it clustered together with *SlRGA4* with 99.5% sequence homology (see Fig. 2, B).

The most interesting results were obtained by analyzing the nucleotide sequence of the 907 bp fragment. It turned out that the difference in length between the fragments obtained with primers NC-LB-9-6678 is due to an insert of the LTR fragment of the retrotransposon of the Ty1-*copia* family, the size of which was 306 bp (Fig. 3). At the same time, the rest of the sequence of the fragment with a size of 907 bp. was 99.7% homologous to the *Ph-3* gene and clustered with it on the dendrogram (see Fig. 3, B). That is, a 907 bp fragment. belongs to the *Ph-3* gene, but with a retrotransposon insertion that disrupts the reading frame and translation of the functional protein. Thus, we found for the first time that the *Ph-3* gene in the tomato genome could have a retrotransposon insertion, which, apparently, renders this gene nonfunctional.

The obtained results confirms the high specificity of the *Ph-3* gene marker Ph3-412 that we created. Most of the analyzed tomato samples (21 out of 24) had the *Ph-3* gene, which was indicated by the presence of the Ph3-412 marker and a 907 bp fragment obtained with primers NC-LB-9-6678. These samples most likely

did not have *Ph-3* homologues, since they lacked a 601 bp fragment, which, according to our data, belongs to the *SlRGA4* homologue, and none of the samples simultaneously had markers 412 bp/907 bp and 601 b.p. Apparently, with the introgression of the *S. pimpinellifolium* genetic material into the tomato genome, the locus containing *Ph-3* replaced the locus containing all homologues of this gene in the *S. lycopersicum* genome via homologous recombination.

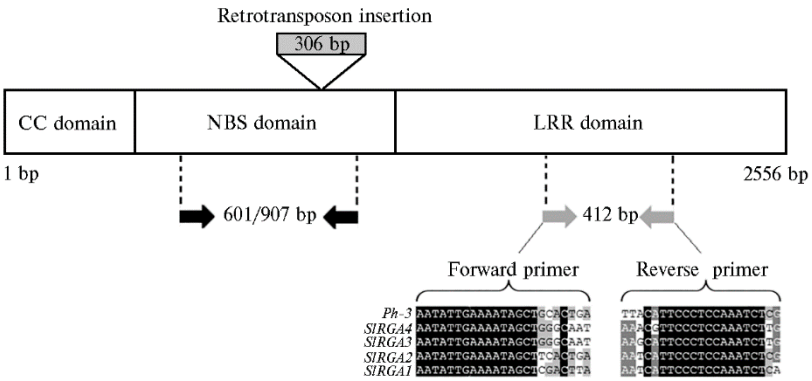

FIg. 3. The tomato (*Solanum lycopersicum* L.) late blight resistance gene *Ph-3*, primer arrangement and insertion of a retrotransposon. CC-domain, NBS-domain and LRR-domain designate the regions of the gene encoding the corresponding domains of the Ph-3 protein. Gray arrows indicate the location of the primers of the Ph3-412 marker, their nucleotide sequences are compared with the nucleotide sequences of the corresponding regions of the gene homologs, black arrows indicate the location of the primers of the marker NC-LB-9-6678; the numbers 1 and 2556 indicate the size of the *Ph-3* gene in bp.

However, samples bearing the Ph3-412 marker appeared to contain an inactive *Ph-3* gene with an insertion of a Ty1-*copia* family retrotransposon. Retrotransposons of this type are very common in the tomato genome (23). The presence of a retrotransposon insert can explain the fact that the Ph3-412 marker we created did not show a clear relationship with the field resistance of the analyzed samples to late blight, they all contained an inactivated form of the *Ph-3* gene.

The resistance of accessions to late blight was due to other genetic factors. For example, the resistant variety Ottawa 30 which, according to our data, has the *Ph-3* gene in an inactive form, contains the resistance genes *Ph-1* and P*h-2* [24, 25]. Since *S. pimpinellifolium* also served as the source of these genes, it can be assumed that in the donor forms included in the selection process, the *Ph-3* gene initially had a retrotransposon insertion. Subsequently, the genetic material of these donors, and not sample L3708, was widely distributed among domestic tomato varieties.

According to our results, for the same reason, the marker NC-LB-9-6678 did not show a connection with field resistance to late blight, since the authors carried out its verification in splitting populations of tomato plants obtained by crossing with the initial donor of the *Ph-3* gene, sample L3708 *S. pimpinellifolium* [20]. As a result, despite its nonspecificity, the NC-LB-9-6678 marker allowed the authors to distinguish the inactive form (907 bp fragment) containing the insert in susceptible genotypes from the functional *Ph-3* form of resistant samples, which upon PCR amplification gives a 601 bp fragment, like other homologues of this gene. Since in the work of D.R. Panthee et al. [20] the L3708 sample which did not contain other homologues acted as a donor of the *Ph-3* gene; in the obtained stable forms, such homologues were also absent and a fragment of 601 bp in size. corresponded to the functional *Ph-3* gene.

Interestingly, D.R. Panthee et al. (20) also noted the presence of stable

forms which, according to the results of the analysis, had both 907 bp and 601 bp fragments, and, along with the *Ph-3* gene, beared the *Ph-2* gene. That is, the inactive form of the *Ph-3* gene can enter the tomato genome when other active *S. pimpinellifolium* resistance genes are introduced into it, since these genes are located in different chromosomes, are inherited unlinked, and are selected independently. It can be assumed that domestic breeders could use forms of *S. pimpinellifolium* containing the *Ph-3* gene with an insert as donors of resistance to late blight; therefore, in our experiments, the marker NC-LB-9-6678 did not work as a marker of resistance to late blight.

Thus, we have created a highly specific marker Ph3-412 of the tomato late blight resistance gene *Ph-3*. We also showed that in the tomato varieties of domestic breeding in the presence of the *Ph-3* gene, there are no other homologues of this gene. In the samples we analyzed, in which the *Ph-3* gene was found, there was a retrotransposon insert. The presence of such an insert can lead to a loss of functional activity, which must be taken into account when marking the *Ph-3* gene during marker-mediated selection for late blight resistance. Tomato forms in which the *Ph-3* gene does not have a retrotransposon insertion should be used as late blight resistance donors. The Ph3-412 marker we developed may be used both in identification of such donors when applied together with the NC-LB-9-6678 marker and in breeding programs.

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