

БИОЛОГИЯ



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TBSV P19 protein as a trigger of salicylic acid-induced resistance of *Solanum lycopersicum*

Abstract: Interaction between a virus and a host plant is of great current interest. In light of this, given article attempts to determine the role of virus proteins in an activation of defence mechanisms associated with salicylic acid in *Solanum lycopersicum* (tomatoes) during Tomato Bushy Stunt Virus (TBSV) infection. The influence of TBSV on tomatoes Money Maker cultivar and the tolerance of the cultivar to an infection were studied. No symptoms were observed during TBSV amplification in Money Maker. Protein P19 was identified as one of the signaling proteins in response to which plants activate a salicylic acid-induced defense mechanism. It might indicate the role of TBSV P19 protein as an initiator of salicylic acid-induced resistance. The obtained results might help to elucidate defence mechanisms of plants from viruses.

Keywords: TBSV, P19 protein, *Solanum lycopersicum*, Salicylic acid, PR-proteins.

INTRODUCTION. Plant resistance to viruses is determined by salicylic acid (SA)-induced defence mechanisms or an activation of RNA-interference (RNAi) process [2]. An activation of SA-induced mechanisms initiate several defense reactions including a generation of reactive oxygen species (ROS), hypersensitive response, synthesis of PR-proteins (pathogenesis-associated proteins) and apoptosis [2, 3]. An infection of *Nicotiana tabacum* with tobacco mosaic virus (TMV) leads to the significant elevation of SA, as well as to the increased expression of PR-genes in both inoculated and apical leaves [5]. Moreover, there was determined a synergetic interrelation between RNAi and SA-induced defence of plants during a viral infection [5, 6]. Interestingly, virus suppressors *Cucumber mosaic virus* (CMV) 2b and *Potyvirus* P1-HcPro interfere with SA-induced defence system of plants. Furthermore, RNA-dependent RNA-polymerase involved in RNAi is induced by a salicylic acid during an infection with *Tobacco mosaic virus* [7]. Thus RNA-interference and SA-induced resistance can functionally interact. Additionally this resistance mechanism can either directly or indirectly be activated by RNA-interference. Virus suppressors also might play a key role in SA-induced resistance [8].

MATERIALS AND METHODS. Plant and virus materials *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*) plants were grown in 250 ml pots in a growth room. Growing conditions are 16/8 h photoperiod under white-fluorescent lamps and temperature regime of 25/20 (day/night) with relative humidity of 80%. For inoculation with virus material carborundum (d = 0,037mm) was applied to the surfaces of 3 middle leaves of 30 days old plants and they subsequently were mechanically inoculated with 50 μ l of material per plant (50 μ l of virus per 200 ml of 10 mM sodium phosphate/biphosphate buffer pH 6.9). Virus-infected and mock-inoculated (with 10 mM sodium phosphate/biphosphate buffer pH 6.9) plants were separated and grown under the same conditions.

Western blot analysis. Protein samples extracted from mock-inoculated and TBSV infected plants were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins

in the gels were electrophoretically blotted onto nitrocellulose membrane (Osmonics, Westborough, MA). Protein transfer efficiency was verified by staining the membranes with Ponceau S (Sigma, St. Louis, MO). The membrane was subsequently used for immunoblotting with the specific antibody raised against P19 TBSV protein. Primary antibodies were diluted 5000-fold and secondary antibodies (alkaline phosphatase conjugated goat anti-rabbit antiserum; Sigma) were diluted 3000-fold. The blots were visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as the substrate.

RNA isolation. Extraction of total RNA was performed according to the protocol set of Total RNA Mini SI Isolation Spin-Kit (AppliChem). Total RNA was isolated from leaves according to the protocol set of Total RNA Mini SI Isolation Spin-Kit (AppliChem). The yield and quality of the RNA was determined by spectral photometry at 260 and 280 nm using the NanoDrop system (Germany).

qPCR. For qRT-PCR, 10 μg of total RNA was used. Oligo-dT primers (5 $\mu g/\mu l$) were used for first strand cDNA synthesis. Reverse transcription was carried out at 40 using M-MuLV RNase H+ (plus) Reverse Transcriptase (M-MuLV RT) according to the manufacturer's instructions (Phusion RT-PCR Kit, Thermo Scientific, Lithuania). Following RT-PCR, the cDNA was treated with ribonuclease H and RNase solution A (Thermo Scientific, Lithuania). The obtained cDNA was diluted 1:10, and PCR was performed in a volume of 15.5 μl of Sybr Green PCR kit (Abgene, Hamburg, Germany) and 9.5 μl of diluted cDNA, using the Applied Biosystems (Darmstadt, Germany) 7500 RealTime PCR System. The PCR conditions were as follows: 1 cycle at 50 ° C for 1 min, 1 cycle at 9 ° C for 3 min, 40 cycles at 90 ° C for 10 s, 60 ° C for 40 s, 72 ° C for 5 min .

In order to find an internal standard for normalization, we generated specific primer sets with Primer Express 2.0 (Applied Biosystems). Primers were constructed on the *Oligo7* software (Table 1). Probes were constructed using SYBR Green and passive reference dyes ROX, FAM.

TABLE 1 – Primer sequences for real-time PCR

Gene	Primer sequence, 5'→3'
p19 – F	CGGCTACATAACGATGAGAC
p19- R	GCATAGTTAACCGAATCTCCC
<i>prp6</i> –F	GTACTGCATCTTCTTGTTTCCA
<i>prp6</i> -R	TAGATAAGTGCTTGATGTGCC
p33-F	CACGAGCACACATGGAGGAT
p33-R	GTGGACGCGATCACCTTAGT
Ubiquitin- F	AGGGATCCCACCAGATCAAC
Ubiquitin - R	GCAGCACACAGGACATTCAC

DISCUSSION. Middle level of leaves of 35 days old tomatoes cv. *Money Maker* were rub-inoculated with WT-TBSV virions as described previously. During 35 dpi (days post inoculation) of an experimental period all plants did not show any symptoms of viral infection such as curling, yellowing of leaves and stunting (Fig 1). Viral protein accumulation was analyzed by Western blot assay of P19 protein (Fig 2).

Four plant categories are distinguished based on the degree of resistance: sensitive, tolerant, supersensitive and extremely resistant. The diagnosis of a viral infection by western blotting assay of the viral protein P19 was carried out in order to determine a category of above-mentioned cultivars of tomatoes. The suppressor protein P19 is an indicator of a viral infection. Western blot analysis was performed according to the protocol using polyclonal antibodies against P19 (Fig. 2). Apical leaves of tomatoes were used for an experiment.

On the left - TBSV-infected *N. benthamiana* (*positive control*); on the right TBSV-infected tomatoes cv. *Money maker*

Note – an equal amount of proteins (20 $\mu g/ml$) was loaded into an each well. Comparative intensity values correspond to the respective formazan bands of the protein P19 (upper row – 38 kDa proteins, lower row – P19). Images were processed using ImageJ software.



FIGURE 1 – Symptom development of TBSV infection on tomatoes cv. Money Maker
 A – Plants before inoculation. B – Plants after 21 days post inoculation (dpi). On the left mock - inoculated plant, on the right TBSV- inoculated plant

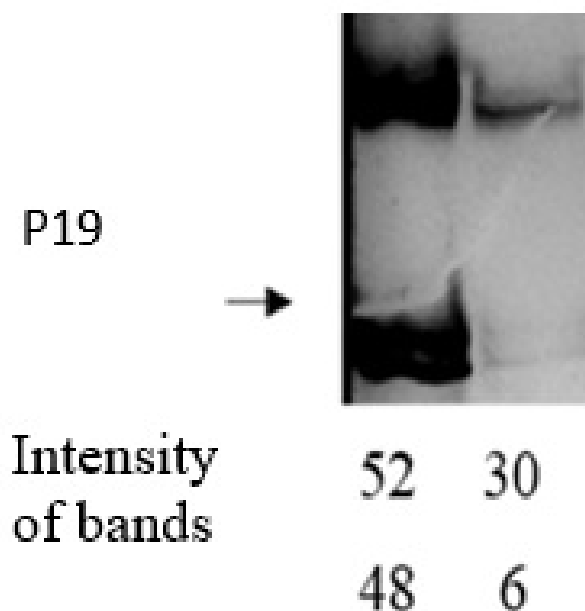


FIGURE 2 – Western blot analysis of P19 protein

The results of experiments demonstrate a tolerance of Money maker cultivar of tomatoes to an infection since despite an accumulation of the protein P19 in TBSV-infected plants, no apparent symptoms were detected.

An influence of TBSV infection on *N. benthamiana* and *Money maker* tomatoes cultivar was studied by mechanically inoculating 30 days old plant leaves.

Balachandran S. et al. (1997) showed that an early activation of defence mechanisms of *N. tabacum* against tobacco mosaic virus (TMV) leads to local damages of photosynthetic apparatus, resulting in a malfunction of photosynthetic parameters [9]. Moreover, a viral infection inhibits an expression of genes associated with functions of chloroplasts and photosynthesis [10].

Due to the fact that in the previous experiment during TBSV infection no visible symptoms and changes in the level of photosynthesis were observed, whereas the virus protein P19 was expressed, it was decided to study other parameters of the photosynthesis. Virus-infected plants demonstrate pronounced morphological and physiological changes in the form of symptoms, such as leaf chlorosis and necrosis, associated with alterations in the structure and function of the chloroplast [11].

In previous studies Chl-FI were used for an early diagnosis of a tobacco mosaic virus (TMV) infection in virus-infected *N. tabacum*. It has been shown that the early sign of an infection is the reduction in the level of fluorescence intensity and the light-dependent development of the

chlorotic-mosaic symptoms [12]. Similar results were obtained by M.L. Perez-Bueno (2006) by studying the effect of *Pepper mild mottle virus* (PMMo-V) on *N. benthamiana* [13]. According to the study, changes in the structure of the chloroplast and a corresponding decrease in the efficiency of photosynthesis have been established.

To date, more than 100,000 individual compounds of secondary metabolism have been identified. The secondary metabolism compounds, in contrast to primary metabolites, have a functional significance not at the cell level, but at the level of the whole plant. Secondary metabolites include alkaloids, terpenoids, phenolic and many other compounds. The group of phenolic compounds contains a couple of dozen members, including a salicylic acid [14]. Salicylic acid is one of the important signalling molecules in plants involved in the antiviral defense mechanism. Thus, it is of interest to study the effect of viral proteins on the accumulation of salicylic acid in plants during viral infection. For this purpose, an experiment was performed using *in vitro* synthesized transcripts of TBSV mutants (Fig. 3):

1. Mutant TBSV – RMJ, which does not express the capsid protein due to a complete elimination of the translatable sequence of its gene and with the incorporated gene of the GFP-reporter protein;
2. Mutant TBSV – 157, which does not express the protein P19.

30-35 days old Money maker (MM) tomatoes plants were inoculated with TBSV- RMJ, TBSV-WT, TBSV-157 transcripts. Control plants were inoculated with phosphate buffer without viral RNA. Total RNA was extracted at 14 dpi according to the protocol (Fig. 4) and cDNA was synthesized using RT-PCR. RNA concentration was measured on the NANO Drop spectrophotometer.

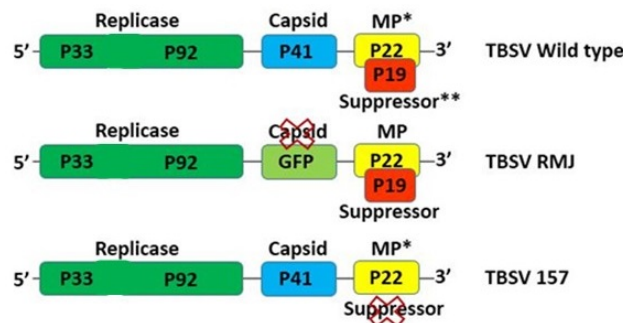
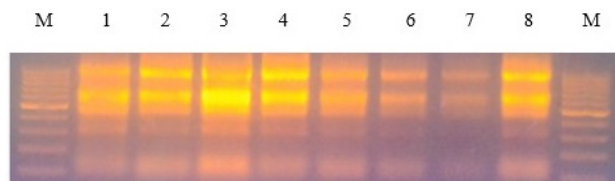


FIGURE 3 – schematic representations of genomes of wild-type TBSV and modified mutants obtained by site-directed mutagenesis

Note - TBSV (*Tomato Bushy Stunt Virus*) encodes the conservative suppressor protein P19. TBSV is a typical representative of the *Tombusviridae* family and contains a sense single-stranded molecule of 4800 nucleotides enveloped in the 41 kDa capsid of 180 identical protein subunits. The virus encodes five proteins: replicases P33 and P92 are translated by genomic RNA (gRNA), a capsid protein P41 by sub-genomic RNA1 (sgRNA1), suppressor protein of nuclease activity P19 and protein responsible for the movement of the virus in the host P22 by sub-genomic RNA2 (sgRNA2) [15].

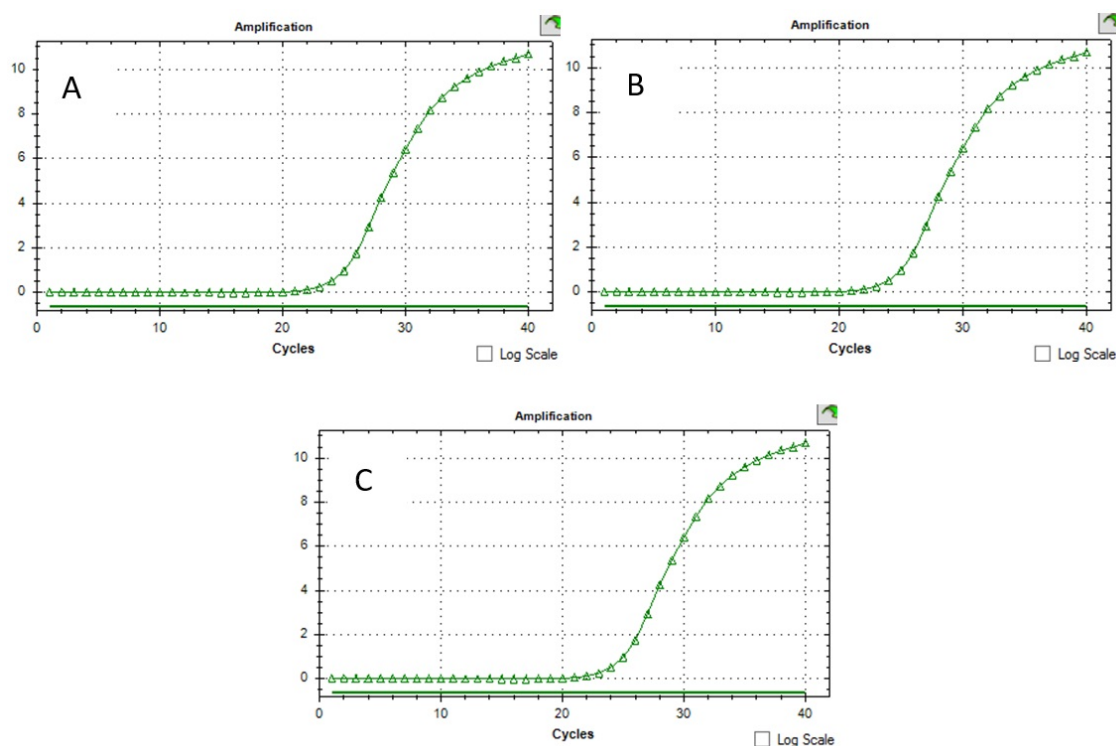


M – 100 kB molecular marker; 1 - a control plant; 2 - a plant inoculated with WT virus; 3 - a plant inoculated with RMJ-mutant; 4 - a plant inoculated with 157-mutant

FIGURE 4 – A detection of total RNA of Money Maker on 1% agarose gel

Real-time PCR was performed for 3 genes: two viral – *p19* (a factor of pathogenicity), *p33* (a viral replicase) and *prp-6* (a marker gene of SA). A housekeeping gene *ubiquitin* was used as a positive control. The experiment was repeated twice. The results of PCR analysis of control samples are shown below (Fig. 5).

20-21 nucleotides long forward and reverse primers with an annealing temperature interval of 0,6° C (54,9 - 55,5° C) for genes *p19*, *p33* and *prp-6* were selected on the *Oligo7* software. Products of selected primers were verified using the *BLAST* software. A synthesis of real-time PCR primers and PCR analysis were conducted at the laboratory of organic synthesis of the National centre of biotechnology in Astana.



A – Accumulation of protein p19; B - Accumulation of protein p33; C - Accumulation of protein PRP-6. Δ - ubiquitin; \circ - an analysed gene

FIGURE 5 – Accumulation of proteins in the control samples of MM tomatoes

As expected, control samples did not express *p19*, *p33* and *prp-6* but only a housekeeping gene *ubiquitin*. Further TBSV-infected samples were analysed.

The results for the samples inoculated with WT-TBSV transcripts are shown below (Fig. 6).

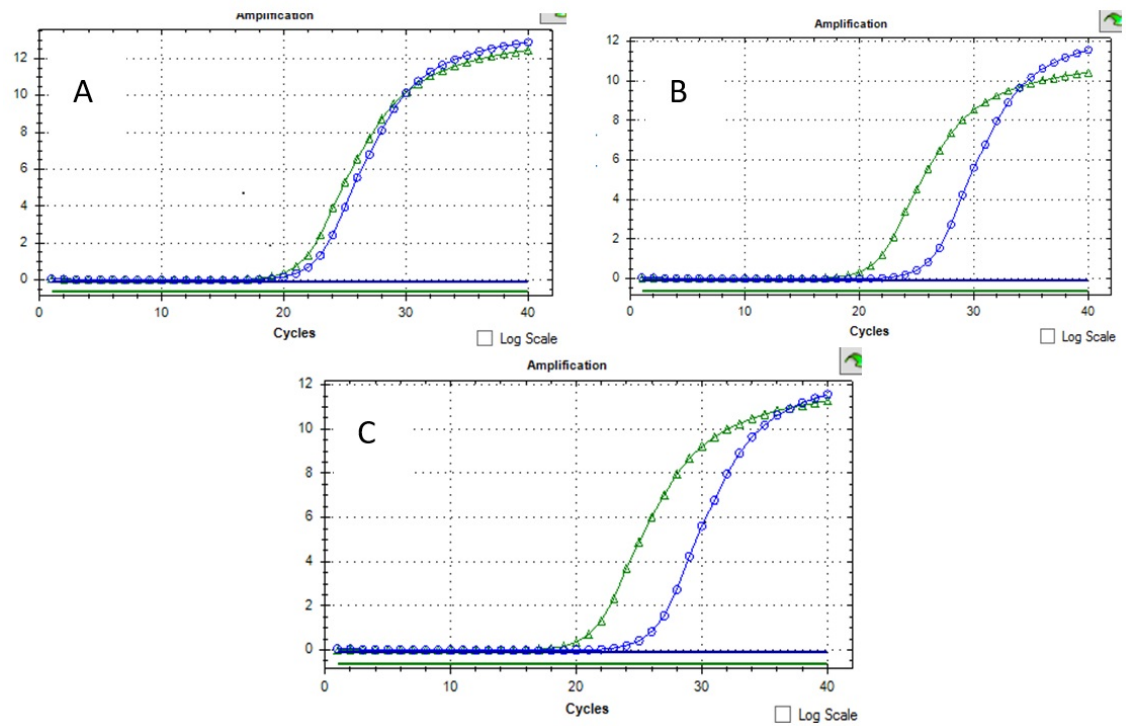
All of the viral proteins including PRP-6 are expressed in the WT-TBSV-infected samples.

The results for the RMJ-infected tomatoes samples are shown below (Fig. 7).

All of the viral proteins including PRP-6 are also expressed in the RMJ-infected MM tomatoes samples. An expression of viral proteins in these samples indicates an accumulation of viral infection. From obtained data it can be seen that the marker gene of SA-induced defence mechanisms *prp-6* is accumulated during viral infection.

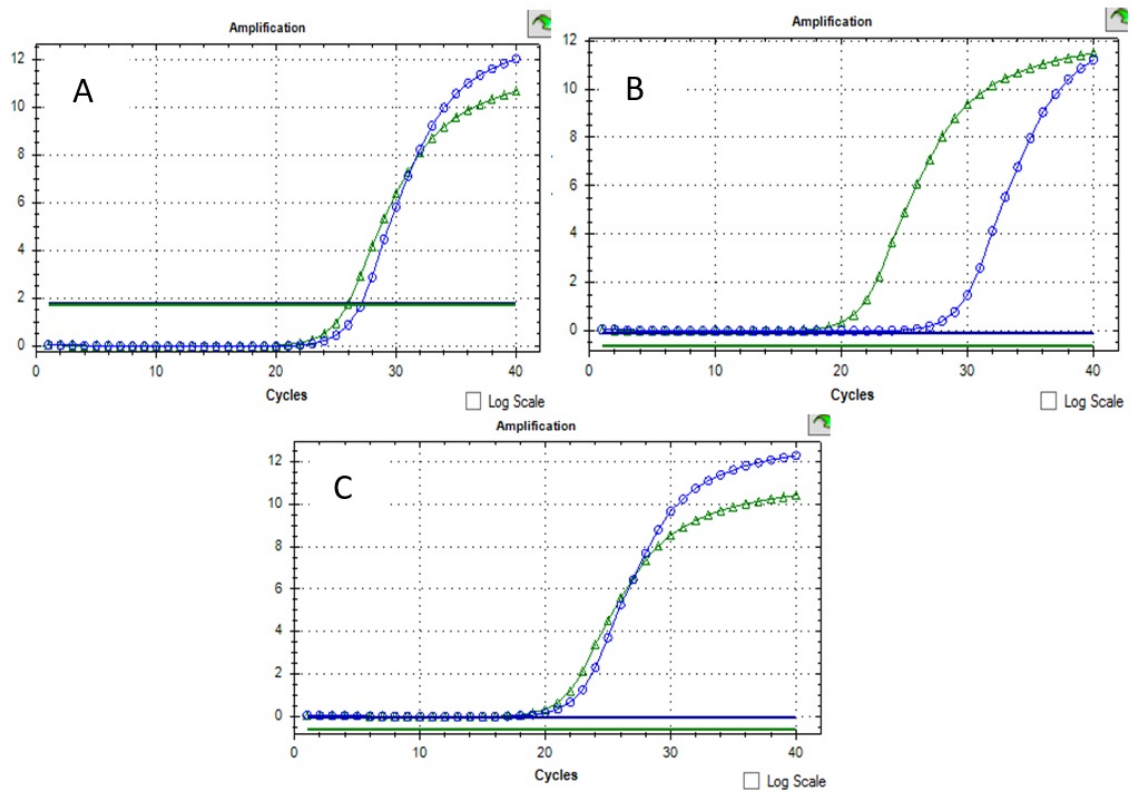
The results for the samples inoculated with 157-TBSV transcripts are shown below (Fig. 8).

All of the viral genes except *prp-6* are expressed in the samples inoculated with 157-TBSV transcripts. It is known that RMJ-mutant does not express the capsid protein due to a total elimination of the translatable sequence of its gene but contains a complete sequence of protein P19. Whereas 157-mutant does not express the protein P19 due to the presence of a stop-codon after an initiation site of *p19* gene. Therefore although PCR analysis demonstrates an expression of *p19* gene, functional protein P19 is not synthesized.



A - Accumulation of p19; B- Accumulation of p33; C - Accumulation of PRP-6. Δ - ubiquitin; \circ - an analysed gene

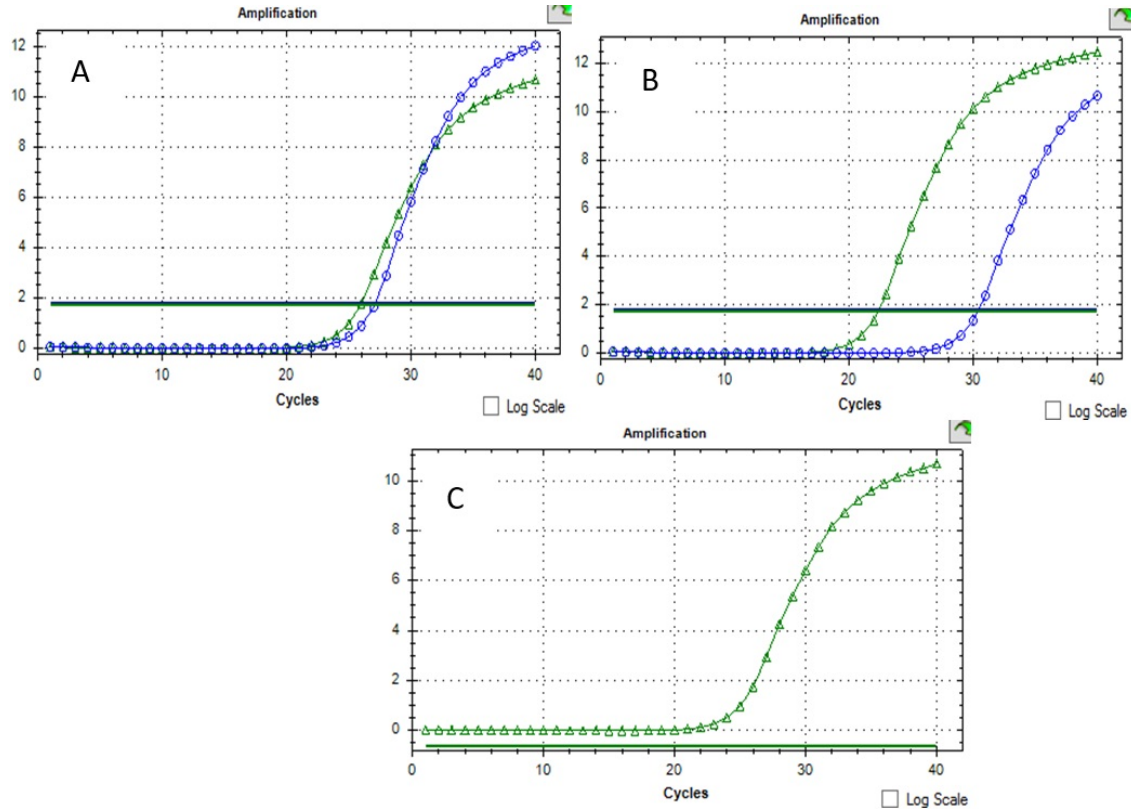
FIGURE 6 – The results of real-time PCR. An accumulation of proteins in the WT-TBSV-infected samples of MM tomatoes



A - Accumulation of p19; B - Accumulation of p33; B - Accumulation of PRP - 6. Δ - ubiquitin; \circ - an analysed gene

FIGURE 7 – The results of real-time PCR. An accumulation of proteins in the RMJ-TBSV-infected samples of MM tomatoes.

From these two mutants RMJ has greater ability of infecting *N.benthamiana*. It is determined by the total absence of capsid protein. The result of the lack of expression of protein P19 in the 157-mutant is the inability to suppress the mechanism of RNA interference of the host due to the weakened interaction with siRNA. This leads to a rapid recovery of the plant.



A - Accumulation of p19; B- Accumulation of p33; C- Accumulation of PRP-6; Δ - ubiquitin; \circ - an analysed gene

FIGURE 8 – The results of real-time PCR. An accumulation of proteins in the 157-TBSV-infected samples of MM tomatoes

According to real-time PCR data obtained from an analysis of a viral protein expression, it was found that the protein replicase P33 and the protein-suppressor of RNA interference P19 are expressed in all virus-infected samples, but not in the control plants inoculated with phosphate buffer. And the marker gene of the SA-dependent defence pathways PRP-6 is expressed only in the samples inoculated with transcripts of WT-TBSV and RMJ-TBSV which contain the P19 protein, but not in the samples with the lack of the protein P19.

Our results demonstrate that the protein P19 is one of the signalling proteins in response to which plants activate a SA-induced defense mechanism. That is, one of the functions of the TBSV protein P19 can be an initiation of SA-induced resistance.

The results of several studies support our obtained data. Plants developed a multifaceted immune system, including an effector-triggered immunity (ETI), to protect themselves from fungal, bacterial and oomycete pathogens [16, 17]. Many studies have shown that the SA serves as a key signalling molecule for the activation of the PAMP-triggered immunity (PTI) after infection with these various types of pathogens [18, 19]. For comparison, the mechanism by which plants resist the virus infection is less known. Because the viruses are intracellular pathogens and do not generate extracellular PAMP, they cannot initiate PTI, which is activated by PAMP-recognising receptors with extracellular recognition or binding domains. However, a number of plant resistance proteins (R-proteins) have been identified that recognize specific viruses. Similar to many of the R-proteins that recognize pathogens like bacteria, fungi and oomycetes, antiviral R-proteins belong to the class of nucleotide-binding site-leucine-rich repeat (NBS-LRR). They initiate the ETI and SAR via SA-dependent pathways that are identical to pathways activated by non-viral pathogens [20, 21].

In addition to these R-protein-mediated resistance reactions, plants use RNA to combat viral infection. Activation of this cellular process by viral double-stranded RNA leads to a targeted destruction of transcripts generated by both RNA and DNA viruses [22]. There have been some observations that the expression of RNA-dependent RNA polymerase 1, a critical component of the RNA suppression mechanism, is induced by SA in many plants [23] and the expression of the SA-induced gene is suppressed by some of the viral suppressor proteins [24]. Other studies have shown that resistance to various viruses, but not to bacteria or fungi, is mediated via the SA-induced activation of the mitochondrial alternative respiratory pathway [238], although this mechanism has not yet been determined. Together, these data show that the ability of SA to suppress all three major stages of a viral infection, including replication, cell-to-cell movement and long-distance movement [25], depends on its parallel activation of several antiviral defence mechanisms.

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TBSV P19 ақуызы *Solanum lycopersicum* өсімдігінің салицил қышқылымен белсендендірілетін қорғаныс механизмінің триггері ретінде

Аннотация: Бүгінде вирус пен оның қожайын өсімдігі арасындағы өзара әрекеттесуін зерттеу үлкен қызығушылық тудырады. Сол себепті осы мақалада TBSV-инфекциясы кезінде *Solanum lycopersicum* (қызанақ) өсімдігінің салицил қышқылымен байланысты қорғаныс механизмдерінің белсендірілуінде вирустық P19 ақуызының рөлін зерттелді. TBSV инфекциясы кезінде *Solanum lycopersicum* Money Maker сұрыбында ешқандай ауыру белгілері байқалған жоқ. P19 ақуызының *Solanum lycopersicum* Money Maker сұрыбында салицил қышқылы арқылы туындайтын қорғаныс механизмін белсендіретін қосымша ролі анықталды. Алынған нәтижелер вирусқа төзімді өсімдіктер сұрыптарын алу үшін көмектеседі.

Түйінді сөздер: TBSV, P19 ақуызы, *Solanum lycopersicum*, салицил қышқылы, PR протеиндері.

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P19 белок TBSV в качестве триггера индуцированной салициловой кислотой резистентности *Solanum lycopersicum*

Аннотация: Изучение взаимодействия между вирусом и растением-хозяином представляет большой интерес. В свете данной статьи посвящена изучению роли вирусных белков в активации защитных механизмов, связанных с салициловой кислотой в *Solanum lycopersicum* (томаты) во время TBSV -инфицирования. Изучено влияние TBSV на томаты сорта Money Maker и определена толерантность сорта к инфекции. Во время TBSV-инфицирования в томатах сорта Money Maker не наблюдалось никаких симптомов. Белок P19 был идентифицирован как один из сигнальных белков, в ответ на которые растения активируют защитный механизм, вызванный салициловой кислотой. Это может указывать на дополнительную роль P19 белка TBSV в качестве инициатора защитных механизмов растений, связанных с салициловой кислотой при вирусной инфекции. Полученные результаты могут помочь понять способы защиты растений от вирусов для создания вирусостойчивых сортов растений.

Ключевые слова: TBSV, P19 белок, *Solanum lycopersicum*, салициловая кислота, PR-белки.

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