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FINAL REPORT

ANALYSIS OF MOLECULAR BACKGROUND OF LEAF DEVELOPMENTAL ABNORMALITY CAUSED BY VIRUS INFECTION

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Abbreviations

AGO: Argonaute protein
ARF4: auxin response factor 4
CMV: Cucumber mosaic virus
dsRNA: double stranded RNA
DCL: DICER-LIKE
GFP: green fluorescens protein
miRNA: microRNA
nt: nucleotide
Nt-CMV :CMV strain from *Nicotiana tabacum*
RDR6: RNA-dependent RNA polymerase 6
RNAi: RNA interference
sRNA :small RNA
siRNA: short interfering RNA
ta-siRNA: trans-acting siRNA
Trk7-CMV: CMV strain isolated from *Trifolium repens*
ScL-CMV: CMV strain isolated from *Scopolia carniolica*
UTR : untranslated region
VRS : viral RNA silencing suppressor

Summary

There is an evolutionary arms race between plants and viruses. Plants are able to sense the double-stranded RNA molecules derived from the replication intermediers of the invading virus and dice them with DCL enzymes. During the antiviral, silencing process small RNA molecules (21-24 nt long siRNAs) are generated. These viral siRNAs can incorporate into AGO complexes and can target complementer viral sequences for degradation. In plants endogenous small non-coding RNA molecules can also derive from different RNA silencing pathways and regulate gene expression, genome integrity and stability. Cucumber mosaic virus (CMV) is a single-stranded positive sense RNA plant virus, that causes enormous agronomic losses in many crops and it has one of the broadest host range among plant viruses. CMV strains are classified into two subgroups, strains in subgroup I induce severe symptoms while strains belong to subgroup II cause milder symptoms, generally. In our experimental system tomato plants were infected with two different CMV isolates: Trk7-CMV and ScL-CMV. Although both isolates belong to subgroup II, Trk7-CMV causes mild mosaic symptoms, ScL-CMV causes severe shoestring symptoms on leaves. ScL-CMV infected leaves have very narrow leaf lamina and dorso-ventral symmetry of leaf is dramatically changed. The main goal of the study was to investigate the molecular

background of shoestring symptom of tomato caused by a ScL-CMV strain, including both plant and viral factors.

To identify the viral genomic region which is responsible for development of shoestring symptom, reassortants and recombinants were made from Trk7-CMV and ScL-CMV strains. After symptom analysis of infected tomatoes we found that 3' region of RNA2 is responsible for the shoestring symptom development. This region contains the 2b silencing suppressor and 3'UTR of the virus. To identify the interacting partner of 2b protein, 2b silencing suppressors from different CMV isolates (ScL-CMV, Trk7-CMV, Nt-CMV) were FLAG tagged, cloned and transformed into tomato plants (*Solanum lycopersicum cv.Moneymaker)*. T0 tomato plants were analysed and 2b gene expression was proved in the case of all constructions. Transgenic tomato plants expressing ScL-CMV 2b showed leaf phenotype similar to the shoestring symptom caused by ScL-CMV. Silencing activity of ScL-CMV 2b and Trk7-CMV 2b were further analysed by argoinfiltration assay. ScL-CMV 2b protein silencing suppressor activity was enormously stronger than Trk7-CMV 2b not only in local but also in systemic level. This is very interesting because these silencing suppressors are very similar only three amino acid differences were identified at position 14, 35 and 81. To determine which amino acid is important for symptom development, amino acids in 14., 35. and 81. positions were changed one by one to amino acids of the other isolates at the same position. Silencing suppressor activity of 2b mutants were analysed also by agroinfiltration assay. Based on our results 14. and 35. amino acid is important for the symptom development. Small RNA (sRNA) and double stranded RNA (ds) binding activity of ScL-CMV 2b suppressor was also tested. Based on our results ScL-CMV 2b suppressor - to our suprise - can not bind sRNA and dsRNA. Therefore, we will try to identify the protein interactor partner(s) of ScL-CMV 2b silencing suppressor in the future months.

Shoestring symptom is very similar to the phenotype of an RNAi mutant (rdr6) tomato. The gene product of RDR6 is essential for the production of ta-siRNAs. There is a possibility that the virus interfere with the plant endogenous small RNA system. We found tasiR-ARF4 level was decreased, miR390 and ARF4 were elevated in ScL-CMV infected tomato compared to the Trk7-CMV which causes mild symptoms. Based on our studies we believe that the ScL-CMV 2b inhibit the production of tasiR-ARFs therefore they are not able to repress ARF4 and results in abnormal leaf development. This is in line with previous finding that ARF4 overexpression causes leaf narrowing phenotype in tomato.

The results of this research were continuously presented on international (2 posters) and national conferences (one poster and one presentations).

Posters in international conferences:

Anita Sós-Hegedűs, Pál Salamon, Katalin Nemes, Katalin Salánki, **György Szittya**. Analysis of molecular background of leaf developmental abnormality caused by virus infection. Hungarian Molecular Life Sciences 2017, 31 March – 2 April 2017, Eger (Programme & Book of abstracts, 223. oldal. ISBN 978-615-5270-34-5) poster

Sós-Hegedűs A, Nemes K, Tóth T, Gyula P, Salamon P, Salánki K, Szittya G. Molecular background of virus induced leaf developmental abnormality. IGC Symposium 2017 on Plant RNA Biology, 27-28 September 2017, Oeiras, Portugal. poster

Poster and presentation in national conferences:

Sós-Hegedűs Anita, Gyula Péter, Tóth Tamás, Szittya György. A CMV 2b fehérje működésének vizsgálata szerkezeti modellek alapján. Fiatal RNS Kutatók Fóruma, 2017. június 23. Gödöllő, NAIK-MBK. előadás

Tóth Tamás, **Sós-Hegedűs Anita** , Nemes Katalin , Gyula Péter , Salamon Pál , Salánki Katalin és Szittya György. (2018). Vírusfertőzés következtében kialakuló levélmorfológiai változás molekuláris hátterének vizsgálata. 64. Növényvédelmi Tudományos Napok, Budapest, 2018.02.20.-21.(poszter)
http://www.magyarnovenyvedelmitarsasag.hu/64NTN/NTN64Kiadvany.pdf

Preparation of a manuscript based on the results on “Analysis of molecular background of leaf developmental abnormality caused by virus infection” is already in progress. The results and figures shown in this report outline the high quality of this study, which is anticipated to be published in a top academic peer reviewed journal with high impact factor.

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1. Introduction

RNA silencing is considered as a primary antiviral defence system in plants (Ding and Voinnet 2007). There is an evolutionary arms race between plants and viruses, so plant viruses have evolved viral suppressors of RNA silencing (VSR) as a counter-defence mechanism against the plant RNA silencing machinery in order to successfully establish infection and invade plants systemically (Ding 2010; Ding and Voinnet 2007; Burgyan and Havelda 2011). These highly diverse VSRs can interfere with many steps of the RNA silencing pathway, including small RNA production, processing, stability and activity of the RNA induced silencing complex (RISC) (Csorba et. al. 2015; Sharma et. al. 2012). Since the antiviral RNA silencing pathway and the components of the endogenous RNA silencing pathways overlaps, VSRs may also has a capacity to interfere with many steps of these pathways. RNA silencing derived plant small RNAs (21-24nt) have two categories based on their biogenesis: small interfering RNA (**siRNA**) processed from perfectly double stranded RNA and microRNA (**miRNA)** derived from ssRNA transcripts that form imperfectly double-stranded stem loop precursor structures. Trans-acting siRNAs (**ta-siRNA**) - special group of siRNA - are derived from long noncoding ssRNAs transcribed from TAS loci. TAS mRNA is cleaved by miRNA and the cleavage product is copied into double stranded RNA by RNA dependent RNA polymerase 6 (RDR6). Dicer4 (DCL4) enzyme cut the dsRNA into 21 nucleotide ta-siRNAs in a phased manner in the miRNA cleavage set register, which loaded into AGO1 complex and direct the cleavage of its target mRNA (Allen and Howell 2010).

Plant leaf is usually a planar organ having dorso-ventral symmetry. The dorsal (adaxial) leaf side faces the light and contains photosynthesizing mesophyll cells and the xylem. The ventral (abaxial) side cells are loosely packed for gas exchange and contain more stomata and the phloem. This adaxial-abaxial leaf polarity is established very early during development and it requires connection with the shoot apical meristem. The formation of adaxial-abaxial side is essential for leaf development because if there is no boundary between the upper and lower side than the leaf blade cannot be formed. Adaxial-abaxial symmetry of the leaf is established through the complex network of different transcription factors and small RNAs. HD-ZIPIII transcription factors (REV, PHB, PHV) express at the adaxial side of leaves and so specify adaxial cell fate. The second pathway in adaxial side contains AS1 a MYB domain transcription factor and AS2 which interact with AS1. On the other hand, KANADI transcription factors specify the abaxial side of the leaf (Szakonyi et al. 2010). ARF3 (ETT) and ARF4 auxin response factor genes are in the same pathway with KANADI genes (Pekker et al. 2005). Two different small RNA pathways are also involved in the regulation of leaf development. MiR165/166 represses the HD-ZIPIII genes and restricts their expression to the adaxial leaf side (Emery et al. 2003, McConell et al. 2001). In other pathway miR390 targets TAS3 long non-coding RNA then it is converted by RDR6 and SGS3 to dsRNA and processed by DCL4 into 21 nucleotides siRNAs. Two of these TAS3 derived siRNAs (tas3D7+ and tas3D8+) are conserved and biologically active (Figure 1.a,b) (Allen et al. 2005), they

repress the expression of auxin response factor 3/4 (ARF3/ARF4). ARFs control the transcription of auxin response genes. TAS3 derived siRNAs generate a gradient across the abaxial/adaxial side of the leaf. These ta-siRNAs (tasiR-ARFs) gradient inhibits the ARF3/ARF4 gene expression in the adaxial domain (Figure 1.c) (Chitwood and Timmermans 2010).

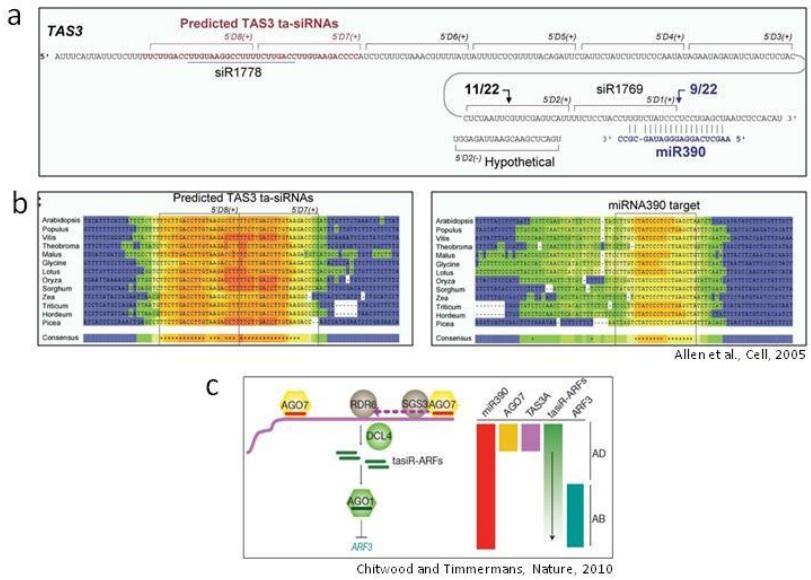


Figure 1. TAS3 non coding RNA and TAS3 derived ta-siRNA pathway. Arabidopsis TAS3 RNA (At3g17185). structure and position of miR390 target site and TAS3 derived ta-siRNAs, predicted functional ta-siRNAs are shown in red**.** (a). Alignment of DNA sequences corresponding to *TAS3* ta-siRNAs tas3D7+ and tas3D8+ and miR390 target site in different plant species (b). TAS3 derived ta-si RNA pathway elements and their localization in adaxial (AD) and abaxial (AB) side of the leaf (c).

Cucumber mosaic virus (CMV) is a single-stranded positive sense RNA plant virus belonging to the genus *Cucumovirus* of the *Bromoviridae* family. CMV strains have one of the broadest host ranges among plant viruses - they infect more than 1200 plant species - cause important agronomic losses in many crops worldwide. CMV strains show high degree of diversity. Different CMV strains can cause strongly different symptoms from mild to severe on the same host plant. These features make CMV an important model for research. Based on the sequence of virus genome CMV strains are classified into two subgroup. Subgroup I strains cause more severe symptoms on their host plants, while symptoms of subgroup II are moderate. The similarity between the two subgroups is around 70-75% (Jacquemond 2012). CMV genome consists of three single-stranded positive sense RNAs and five genes are expressed from them. RNA1 codes 1a protein, RNA2 codes 2a and 2b protein. 1a and 2a proteins are involved in virus replication, 2b protein is the viral RNA silencing suppressor (VSR). RNA3 encodes movement protein and coat protein (Scholthof et. al 2011).

In the last few years it turned out that CMV 2b protein has distinct functions. It is important for symptom development and counteracts with host defence. 2b of several CMV strains interferes with the siRNA accumulation (Diaz-Pendon et al. 2007) and bind small RNA duplexes *in vitro* (Goto et al. 2007). Interaction of 2b protein with host miRNA pathway is

also documented (Lewsey et al. 2007, Lang et al. 2011, Du et al. 2014). 2b of Fny and SD CMV strain was found to interact with AGO1 and blocks AGO1/RISC slicer activity (Zhang et al., 2006; Duan et al., 2012). Interaction of 2b with AGO4 reduces AGO4 access to endogenous target loci and consequently modulates endogenous transcription (Duan et al., 2012; Gonzalez et al., 2010, 2012; Hamera et al., 2012).

Different CMV strains can cause different symptoms on infected tomato plants. Cillo et al. used two CMV strains for they work and showed that CMV-LS (belonging to subgroup II) causes moderate leaf blade reduction in tomato, while CMV-Fny (belonging to subgroup I) causes severely altered leaf morphology. They identified a region on the 3' end of RNA 2 containing the 2b protein and the terminal region, which was responsible for the differences of viral symptoms. It was also shown that both CMV strains provoked significant changes in the expression of several transcription factors having a role in leaf morphogenesis (mild increase in the RNA level of HD-ZIP and TCP4/LANCEOLATA, and 4 times increase in the expression level of AS1/PHANTASTICA), and corresponding miRNAs that post-transcriptionally regulate their turnover, over accumulated specifically during CMV-Fny infections but not during CMV-LS infection (Cillo et al. 2009).

In our experimental system we used three different CMV strains. ScL-CMV (from *Scopolia carniolica*, subgroup II, causes shoestring symptoms), Trk7-CMV (from *Trifolium repens* subgroup II, causes mild mosaic symptoms) and Nt-CMV (from *Nicotiana tabacum* subgroup I, causes dwarfism, mosaic symptoms).

Since, the results of the funded project written here are not published yet, therefore we give a detailed description of our results here.

2. Determining the ScL-CMV RNA region and protein products responsible for the symptom development

ScL-CMV isolated from *Scopolia carniolica* plant in Hungary belongs to subgroup II (Salamon et al. 2011). ScL-CMV induces severe leaf narrowing (shoestring) symptoms on a wide range of *Solanaceae* species (Figure 2.) which is very unusual since virus strains in subgroup II causes mild symptoms.

Figure 2. ScL-CMV infection causes shoestring symptom on a wide range of *Solanaceae* species

Our goal was to identify the genomic region which is responsible for shoestring symptom development in tomato. To map the viral symptom determination region, we needed a CMV strain with high sequence similarity to ScL-CMV however it must cause completely different symptom on tomato. Trk7-CMV strain was the perfect tool for mapping of symptom determining region because ScL-CMV and Trk7-CMV are in the same subgroup, have minor sequence differences and Trk7-CMV causes mild mosaic symptoms on tomato. The CMV genome contains three genomic RNAs. The genomic RNAs of Trk7-CMV strain was replaced one by one with corresponding RNA from the ScL-CMV isolate. We infected tomatoes with the reassortant viruses and after the analysis of viral symptom we found that the RNA2 of ScL-CMV was responsible for the shoestring symptom. After that we planned to identify the RNA2 region which was important for the shoestring symptom formation. Different RNA2 chimeras were constructed, tomato plants were infected with them and the viral symptoms were analysed. We found, that only the ScL-CMV RNA2 3'region which contained the 2b silencing suppressor and 3'UTR (Trk7ScL2b-CMV) in Trk7-CMV background, was responsible for the shoestring symptom development (Figure 3.). The virus level was similar in plants infected with different isolates therefore the symptom severity is solely the consequence of ScL-CMV 2b presence and not the different virus level.

Figure 3. Identification of ScL-CMV viral region responsible for shoestring symptom development of tomato. Trk7-CMV (represented by blue genome), ScL-CMV (represented by red genome) and the recombinant Trk7ScL-CMV genom (top panel), symptoms on tomato plants and CMV level with ethidium bromide stained rRNA as a loading control (bottom panels). Northern analysis was carried out with RNA isolated from mock and infected leaves. Radiolabeled CMV coat protein (CP) was used as probe.

So we concluded that it is very likely that the 2b silencing suppressor presence is enough for the shoestring symptom development. To check this possibility we cloned the 2b coding sequence with an epitope tag (FLAG) from Trk7-CMV, ScL-CMV, Nt-CMV isolates into pBIN61 binary vector. FLAG-taged 2b silencing suppressors and empty binary vector were transformed into tomato plants (*Solanum lycopersicum cv.Moneymaker)* using a protocol by Fernandez et al. (2009). T0 tomato plants were analysed and the expression of the 2b gene was proved in the case of all three construct at least three independent transgenic tomato lines. At this point we decided to continue the experiments only with ScL-CMV 2b, Trk7-CMV 2b transformed tomato plants because Nt-CMV 2b expressing lines died right after the first leaves developed and we couldn't grow them up to harvest seeds. T1 generation of ScL-CMV 2b transformed tomato plants were analysed and the presence of 2b protein was proved by Western blot detecting the 2b fused FLAG epitope with FLAG antibody (SIGMA) (Figure 4.c). Plants expressing ScL2b protein showed leaf phenotype which was very similar to the shoestring symptom caused by ScL-CMV (Figure 4.) which means that the leaf laminas were extremely narrow (Figure 4.b). The ScL-CMV 2b transgenic tomato plants had more branches and they were a little bit bigger than the control plants (Figure 4.a). The flowers had narrower petals – this feature is also could be observed during virus infection – but tomato fruits were similar to control plant's fruit and had seeds (data not shown). So we concluded that only the expression of ScL-CMV 2b protein was sufficient to mimic the symptoms of ScL-CMV infected tomato.

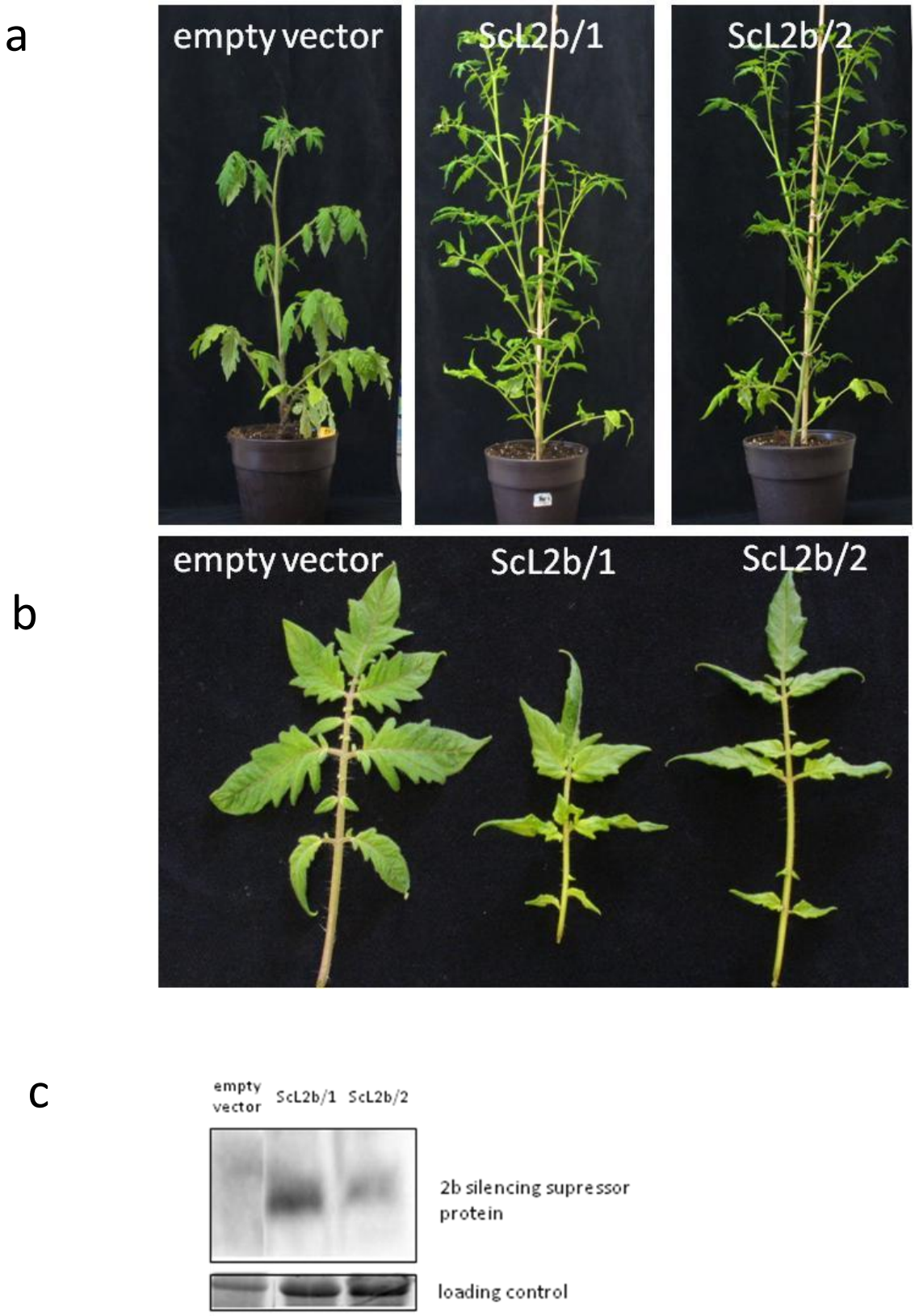


Figure 4. Tomato transformation with 2b silencing suppressor of ScL-CMV isolate. Control (empty vector) tomato plant and two independent tomato lines (ScL2b/1 and ScL2b/2) expressing ScL-CMV 2b protein, whole plant (a) and leaves (b). Western blot analysis of ScL2b/1 and ScL2b/2b tomato lines with FLAG antibody (c).

To find out more the function of CMV 2b proteins, cloned 2bs from Trk7-CMV, ScL-CMV isolates and p19 a well characterized silencing suppressor from Cymbidium ringspot virus (CymRSV) silencing activity were analysed in agroinfiltration test. In these experiments *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* C58C1 containing the following constructs:

- GFP with empty vector
- GFP with 2b of ScL-CMV
- GFP with 2b of Trk7-CMV
- GFP with p19 of CymRSV (well characterised silencing suppressor with 21 nt long small RNA binding activity and strong suppressor activity)

All genes (GFP and silencing suppressors) were driven with constitutive CaMV 35S promoter. GFP expression was detected by UV light. 4 days after infiltration with GFP and empty vector the expression of GFP decreased because the plant's silencing machinery degrades the GFP mRNA. However if the GFP expressing vector were co-infiltrated with Trk7-CMV 2b or ScL-CMV 2b or p19, then the viral silencing suppressor protected the GFP mRNA from degradation. 6 days after the co-infiltration only p19 and ScL-CMV 2b protected the GFP mRNA which means that the ScL-CMV 2b viral silencing suppressor activity is strong, similarly to p19 (Figure 5.).

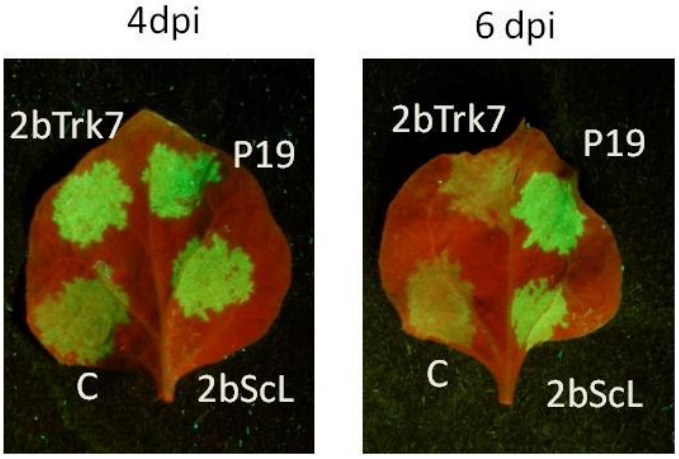


Figure 5. Silencing suppressor activity analysis of different CMV strains. Binary plasmid with constitutive GFP expression was co-infiltrated with empty vector (C), Trk7-CMV 2b (2bTrk7), ScL-CMV 2b (2bScL) and CymRSV p19 (p19) expressing vector. GFP expression was detected 4 and 6 days post infiltration (4dpi, 6dpi) under UV illumination.

Beside the analysis of local silencing activity we also examined the systemic silencing activity of the CMV subgroup II 2b silencing suppressors. In case of local silencing we used wild type *N. benthamiana* plants however for the analysis of the suppression of systemic silencing we used the 16c transgenic line of *N.benthamiana* which constitutively express GFP under the control of 35S promoter. If we agroinfiltrate these plants only with a GFP expressing construct, the GFP transgene will be silenced not only in the infiltrated patch but several days after the infiltration, also in other parts of the plants . After agroinfiltration siRNAs are

generated from the GFP mRNA and these siRNAs can move in the plant for a long distance and act as silencing signals and silence the GFP transgene. Systemic silencing can be monitored easily because the colour of the silenced region will be red under the UV light because the GFP degraded and only the chlorophyl autofluorescence can be seen. However, if the spread of the silencing signal is blocked then the plant remains light green as a result of continuous GFP expression. So for systemic silencing experiment, we co-infiltrated 16c *N. benthamiana* leaves with *Agrobacterium tumefaciens* C58C1 strains containing the following constructs:

- GFP with empty vector
- GFP with 2b of ScL-CMV
- GFP with 2b of Trk7-CMV
- GFP with p19 of CymRSV

All genes (GFP and silencing suppressors) were driven with constitutive CaMV 35S promoter. 16 days after infiltration with GFP and empty vector, red patches were detected in upper non-infiltrated systemic leaves of *N. benthamiana* plants. In case of infiltration with GFP and 2b of Trk7-CMV we got the same results. So 2b of Trk-CMV can't block the moving of silencing signal. Using GFP with 2b of ScL-CMV and GFP with p19 of CymRSV red patches were not seen in plant. So we concluded that 2b of ScL-CMV just like p19 is very effective viral silencing suppressor which can block the spread of systemic silencing signal (Figure 6.).

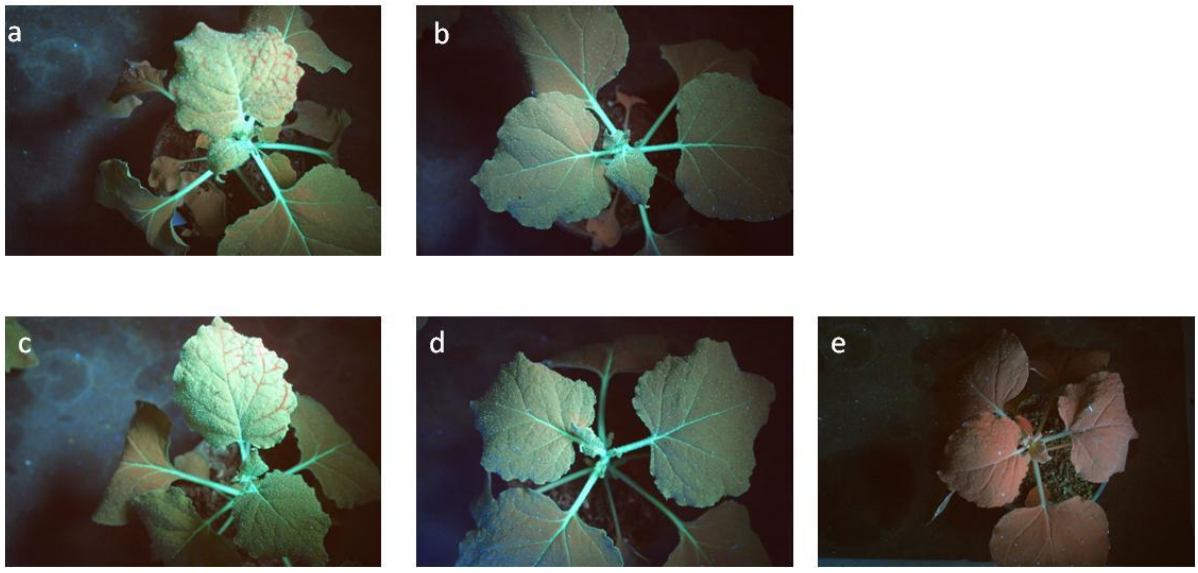


Figure 6. Systemic silencing signal suppression activity of different silencing suppressors. GFP expressing 16c transgenic *N.benthamiana* plants were infiltrated with GFP with empty vector (a); GFP with 2b of ScL-CMV (b); GFP with 2b of Trk7-CMV (c); GFP with p19 (d) at 16 dpi. Small red lines in the veins of green leaves show the speading of systemic silencing signal (a,c). Non transgenic and non treated *N.benthamiana* plant used as a contol to show chlorophyll autoflourescence (e).

The seen enormous difference between the viral silencing suppressor activity of Trk7-CMV 2b and ScL-CMV 2b both in local and also in systemic is very interesting because these viral

silencing suppressors are very similar to each other. There is only three amino acid differences were identified at position 14, 35 and 81 (Figure 7.). In Trk7-CMV 2b the 14. amino acid is histidine the 35. is valine and the 81. is tyrosine. In ScL-CMV 2b the 14. amino acid is glutamine, the 35. is alanine and 81. is phenylalanine. (Figure 7. and Figure 8.)

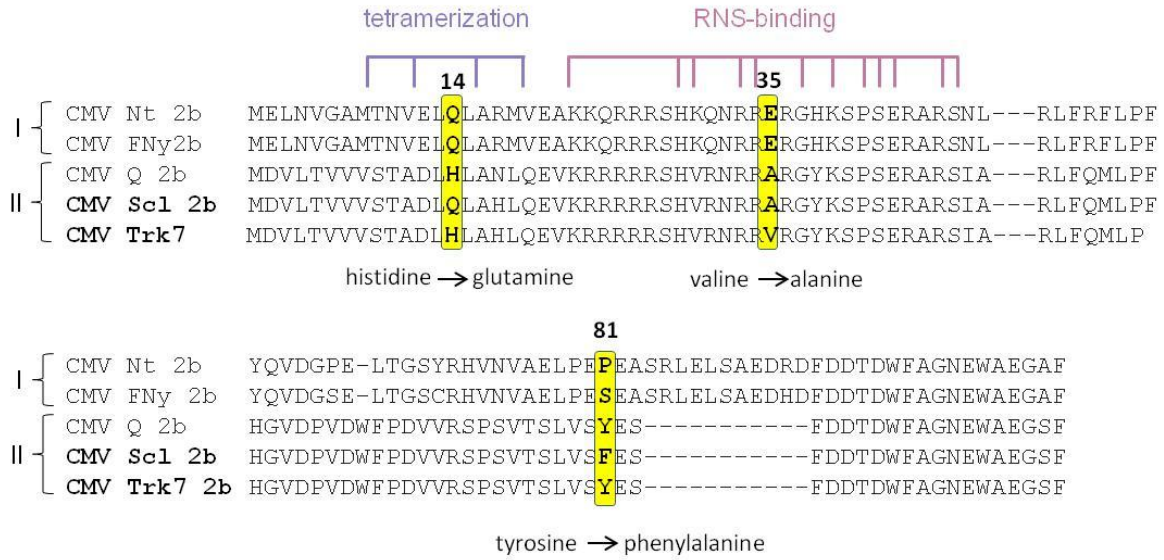


Figure 7 . Amino acid sequence similarity of 2b proteins of CMV strains from subgroup I (I) and subgroup II (II). ScL-CMV and Trk7-CMV differ only in position 14, 35 and 81.

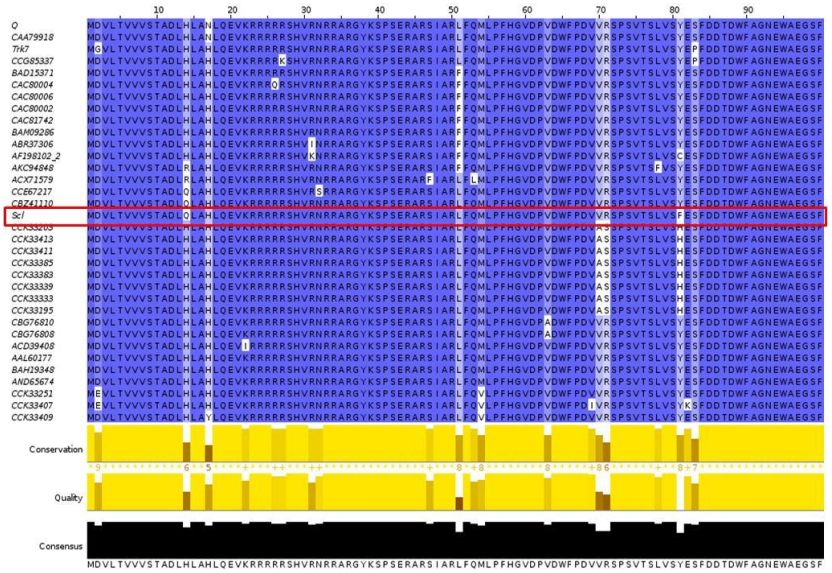


Figure 8. Alignment of amino acid sequence of 2b proteins of CMV strains from subgroup II. Strain names or accession numbers are in the first column.

To determine which amino acid is important for symptom development, amino acids at 14., 35. and 81. positions were changed one by one to amino acids of the other isolates at the same position. Silencing suppressor activity of the 2b mutants were analysed also in agroinfiltration assay (Figure 9.). In this experiment beside the GFP construct we also used a construct which contains two GFP genes in inverted repeat orientation (IR GFP). With the help of this construct the silencing of GFP is accelerated because the transcribed mRNA of IR GFP forms a hairpin structure. This double stranded RNA (dsRNA) induces the silencing machinery very efficiently right after the infiltration so the process will be much faster and stronger compared to the use of a single GFP mRNA expressing construct alone. In the agroinfiltration assays we used the following constructs:

- IR GFP+ GFP and empty vector
- IR GFP+ GFP and 2b of ScL-CMV
- IR GFP+ GFP and 2b of Trk7-CMV
- IR GFP+ GFP and 2b of ScL-CMV glutamine in position 14 changed to histidine (S14)
- IR GFP+ GFP and 2b of ScL-CMV alanine in position 35 changed to valine (S35)
- IR GFP+ GFP and 2b of ScL-CMV phenylalanine in position 81 changed to tyrozine (S81)
- IR GF + GFP and 2b of Trk7-CMV histidine in position 14 changed to glutamine (T14)
- IR GFP + GFP and 2b of Trk7-CMV valine in position 35 changed to alanine (T35)
- IR GFP + GFP and 2b of Trk7-CMV tyrozine in position 81 changed to phenylalanine (T81)

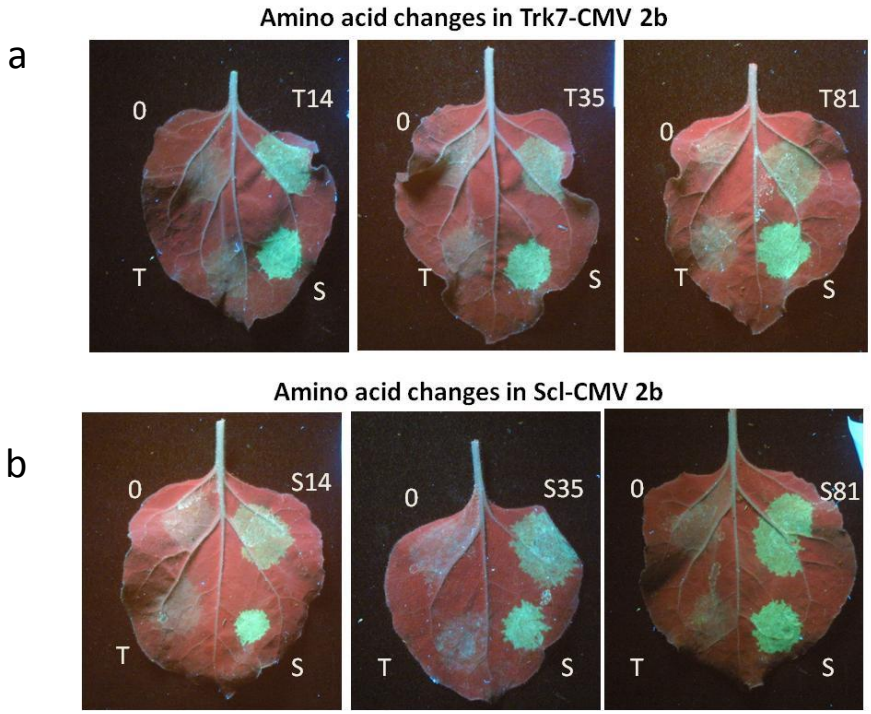


Figure 9. Identification of amino acids of ScL-CMV 2b VSR which is responsible for strong VSR activity. Binary plasmid with constitutive GFP expression, coinfiltrated with plasmid constitutively expressing an inverted repeat GFP mRNA and an empty vector (0) or Trk7-CMV 2b (T), or ScL-CMV 2b (S); or 2b modified in indicated amino acid position (T14,35,81 and S14,S35,S81). GFP expression was detected under UV illumination 4 days post infiltration.

The GFP expression was detected under UV light after 4 days post infiltration. Empty vector (0) and Trk2b silencing suppressor (T) couldn't protect the GFP mRNA coinfiltrated with IR GFP construct, in contrast to ScL2b (S). This observation was in line with our earlier experiments (Figure 5.). If the 14.amino acid of Trk2b silencing suppressor was changed to ScL2b 14.amino acid, the "weak" Trk2b suppressor was transformed to strong suppressor which could protect the GFP mRNA from degradation. Changing of the 35.amino acid of Trk2b had a milder but measurable effect while changing of the 81.amino acid had no effect on the suppressor feature (Figure 9. a). We also did the amino acid changes in ScL-CMV 2b also. If the 14.amino acid of ScL2b silencing suppressor was changed to Trk2b 14.amino acid, the "strong" ScL2b suppressor was transformed to weak suppressor which couldn't protect the GFP mRNA from degradation. Changing of the 35.amino acid had milder and changing the 81.amino acid had no effect on the suppressor activity (Figure 9.b). Based on these results the 14. and the 35. amino acids positions of ScL-CMV 2b are important determinants of the strong VSR function and it is very probable that they have an important role in the symptom development.

3. Characterization of ScL-CMV 2b silencing suppressor

After we identified the amino acids responsible for the strong VSR function we tried to understand, how the ScL2b silencing suppressor interacts with the genetic components of leaf developmental process. Based on the literature we hypothesized that ScL2b might bind small RNAs or long double stranded RNAs or it interacts with a protein component of the RNA silencing machinery.

3.1. Analysis of small RNA binding activity of ScL2b

To test the possibility of small RNA binding of ScL2b, different silencing suppressors (Trk2b, ScL2b, p19) were expressed in agroinfiltrated *N. benthamiana* leaves. Their silencing activity was checked with GFP coinfiltration, VRS assay extracts were taken at 3 dpi from the agroinfiltrated leaf tissues. Protein extracts containing different VRS were incubated with radionucleotide labelled siRNA or miRNA probes (Mérai et al. 2006). Gel mobility shift assay was performed with different samples (Figure 10.). Silhavy et al. demonstrated that P19 strongly bind siRNA (2002) so we used p19 as a positive control in our experiment. Buffer (no plant extract) and pBIN61 were used as a negative controls. In our gel mobility shift assay Trk2b and ScL2b couldn't bind neither siRNA nor miRNA in contrast to p19 which served as a positive control.

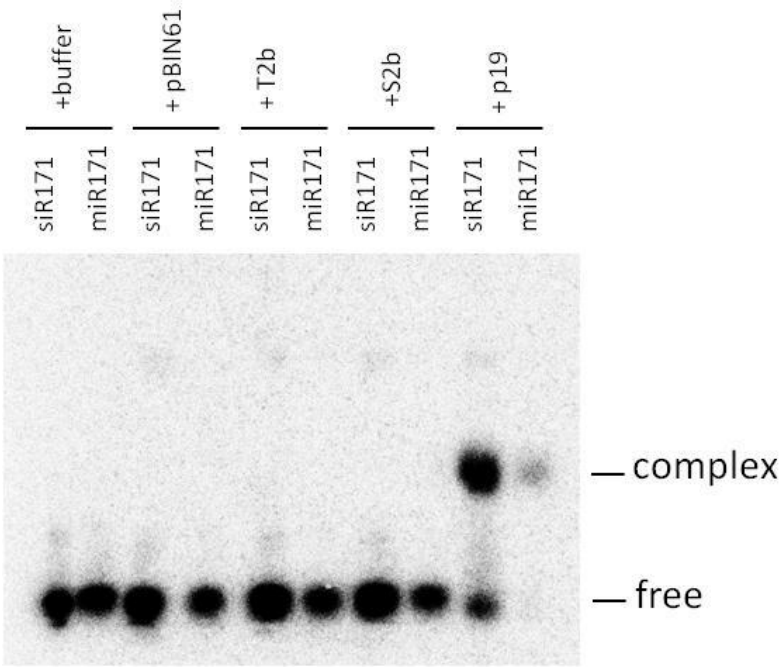


Figure 10. **Gel mobility shift assay with different viral silencing suppressors**. Buffer means no plant extract only buffer, pBIN61 means plant extract from *N.benthamiana* leaves infiltrated with empty pBIN61 vector. Trk2b (T2b), ScL2b (S2b) and p19 means plant extract from *N.benthamiana* leaves infiltrated with the given viral silencing suppressor. Based on the literature data we used radiolabeled siR171 and miR171 for the experiment. siR171 means modified miR171 miRNA with perfectly matched strands.

3.2. Analysis of long double stranded RNA binding activity of ScL-CMV 2b

Viral silencing suppressors have different strategies to inhibit plant silencing suppressor machinery. Long dsRNA binding were documented in case of different viral silencing supressors. In our experiment *N. benthamiana* plant leaves were co-infiltrated with long dsRNA (inverted repeat GFP) and different silencing suppressors expressed in Agrobacteria. Mammalian reovirus outer shell polypeptide sigma3 (σ-3) is among the best-characterized dsRNA binding proteins (dsRBS). σ-3 carries conservative dsRNA-binding motifs and bind dsRNAs *in vitro* and *in vivo* (Denzler and Jacobs, 1994, Huismans and Joklik 1974). σ-3 suppressor was used as a positive control - we expect that it has strong long dsRNA binding activity. P19 with only a strong siRNA binding capacity was used as a negative control. The following constructs were used for the experiment:

IR-GFP + empty vector EV
IR-GFP + σ-3 (σ-3)
IR-GFP + p19 (p19)
IR-GFP + ScL-CMV 2b (S2b)
IR-GFP + Trk-CMV 2b (T2b)

Samples were collected from the infiltrated patches of *N. benthamiana* leaves at 4 dpi (11.a) and 6 dpi (11.b) GFP mRNA and GFP specific siRNA levels were measured by Northern blot and small RNA northern blot analysis. In line with the previous report (Lichner et. al 2003) strong GFP mRNA expression with very low levels of GFP specific siRNA indicated that σ-3

suppressor inhibited GFP silencing because of the binding of long dsRNA (IR-GFP). But in case of p19, ScL-CMV 2b and Trk-CMV 2b no GFP mRNA and big amount of GFP specific siRNA can be detected which means that these silencing suppressors can't bind the long dsRNA.

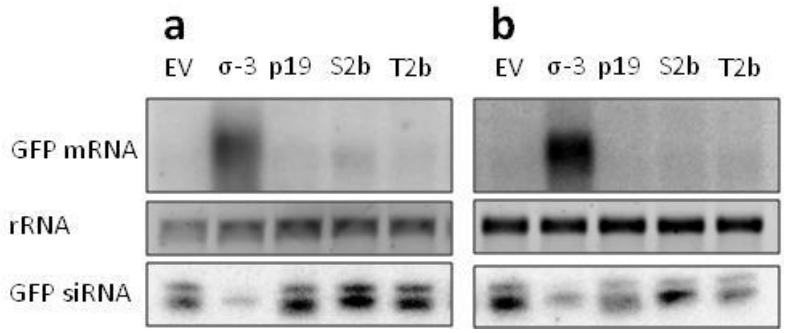


Figure 11. Long dsRNA binding activity of different silencing suppressors. IR GFP was co-infiltrated with different viral silencing suppressors (empty vector (EV), σ-3 (σ-3), p19 (p19), ScL-CMV 2b (S2b), Trk-CMV 2b (T2b)). Levels of GFP mRNA (top panel) and GFP specific siRNA (bottom panel) were measured in 4dpi (**a**) and 6dpi (**b**). Ethidium bromide-stained rRNA is shown as a loading control. Radioactively labelled GFP PCR fragment was used as a probe for northern blot and small RNA northern blot analysis.

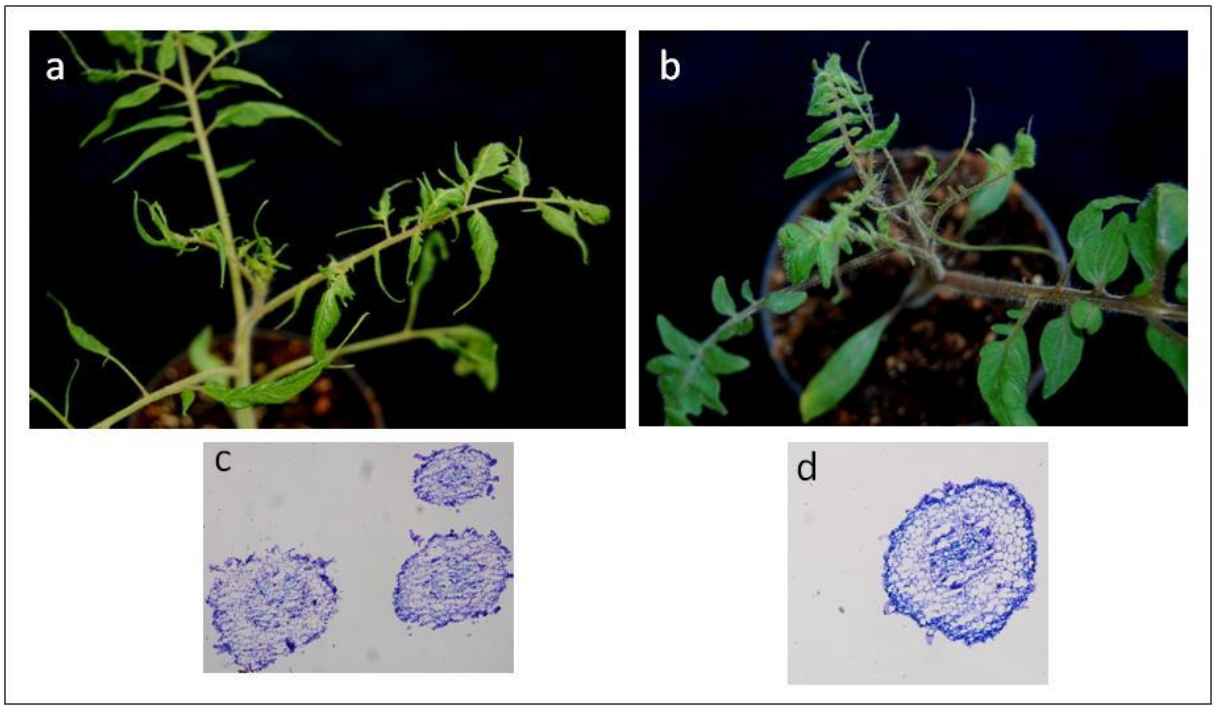
3.3. Interaction with a protein component of the silencing pathway

As ScL-CMV 2b can not bind siRNA/miRNA and long dsRNA, the other possibility, that it has interaction with a protein component of the plant RNA silencing machinery. Based on the literature there are some likely candidates for this interaction. It can interact with AGO1, AGO4 or RDR6 proteins. Examinations of these possibilities by immunoprecipitation are in progress.

4. Investigating leaf developmental abnormality in ScL-CMV infected tomato plants

Yifhar et al. (2012) characterized an interesting tomato mutant. This mutant had extremely narrow leaves that lack leaf blade expansion due to the abnormal leaf development. It was demonstrated that it had a point mutation in RNA-dependent RNA polymerase 6 (RDR6) gene, so this mutant (*rdr6 mutant*) had no functional RDR6. RDR6 gene is essential for the biogenesis of ta-siRNAs and two ta-siRNAs generated from TAS3 RNA named tas3D7+ and tas3D8+ (tasiR-ARFs) are essential in tomato leaf development. TasiR-ARF gradient across the abaxial/adaxial side of the leaf inhibits the ARF3/ARF4 gene expression in adaxial domain (Figure1.c) (Chitwood and Timmermans 2010). In absence of tasiR-ARFs ARF3/ARF4 expression will be elevated in both side (adaxial and abaxial) of the leaf and leaf developmental process will be changed. The resulting tomato leaves will develop a shoesting phenotype (Yifhar et al. 2012).

We observed that ScL-CMV caused symptom resembles to the phenotype of tomato *rdr6* mutant (Figure 12.) so we decided to analyse the components of tasiR-ARFs pathway.



Figures 12. ScL-CMV caused symptom resemble to the phenotype of tomato *rdr6* mutant. ScL-CMV infected tomato *(M82*) plant symtoms (a) and cross section of infected leaves (c). *rdr6* mutant tomato *(M82)* phenotype (b) and cross section of shoestring leaf (d).

We monitored the expression changes of tasiR-ARF pathway components at different time points after virus infection (10, 14 and 27dpi) and with different virus strains. Besides Trk7-CMV, ScL-CMV strains, Trk7ScL2b-CMV recombinant virus strain was also used in experiments. Trk7ScL2b-CMV is a Trk7-CMV strain in which the 2b silencing suppressor and 3' non coding region were changed to ScL-CMV 2b and 3' UTR. Trk7ScL2b-CMV recombinant virus causes shoestring symptoms just like ScL-CMV strain (Figure 3.). After viral infection the symptoms were not visible at 10dpi, but at 14dpi symptom development started and at 27dpi symptoms were very severe (Figure 13.), so we decided to use 14 and 27dpi virus infected leaves for further analysis. Samples were collected form mock and virus infected leaves at 3. and 4. leaf level at 14dpi and 6.-11. leaf level at 27dpi depending on the plant size. From these samples RNA was isolated and virus level was determined by northern blot. We found that at 14dpi the virus level was similar in the case of three CMV isolates and at 27dpi the virus level only in the case of Trk7-CMV strain was a little bit lower (Figure 14.c, d). In small RNA northern blot tasiR-ARF (tas3D7+) and two miRNAs expression patterns were tested (Figure 14.a, b). Tas3D7+ level was decreased at 14 and 27dpi in Trk7ScL2b-CMV and ScL-CMV infected tomato plants compared to Trk7-CMV, so the level of tas3D7+ correlated with symptom severity (Figure 14.a,b). MiR390-5p level was elevated only in 14dpi in Trk7ScL2b-CMV and ScL-CMV strain compared to Trk7-CMV (Figure 14.). Mir390-3p expression was the same that miR390-5p at 14dpi, but, its level didn't show any differences between different samples at 27dpi. The downregulation of Tas3D7+ in tomato plants infected with Trk7ScL2b-CMV and ScL-CMV predicts the upregulation of its target gene ARF4.

To check that the Tas3D7+ targeted ARF4 expression is indeed upregulated we measured its expression level in infected tomato leaves by qRT-PCR. Samples were the same that were used in the experiment in Figure 14. As it was expected, the ARF4 expression level was indeed increased at 14dpi and especially at 27dpi in tomato plants infected with Trk7ScL2b-CMV and ScL-CMV strain (Figure 15.a, b). We believe that the ScL-CMV 2b inhibit the production of tasiR-ARFs therefore they are not able to repress the expression of ARF4 in the adaxial part of the leaf and as a consequence shoestring leaves are formed during the virus infection. This is in line with previous finding that ARF4 overexpression causes leaf narrowing phenotype in tomato (Yifhar et. al 2012).

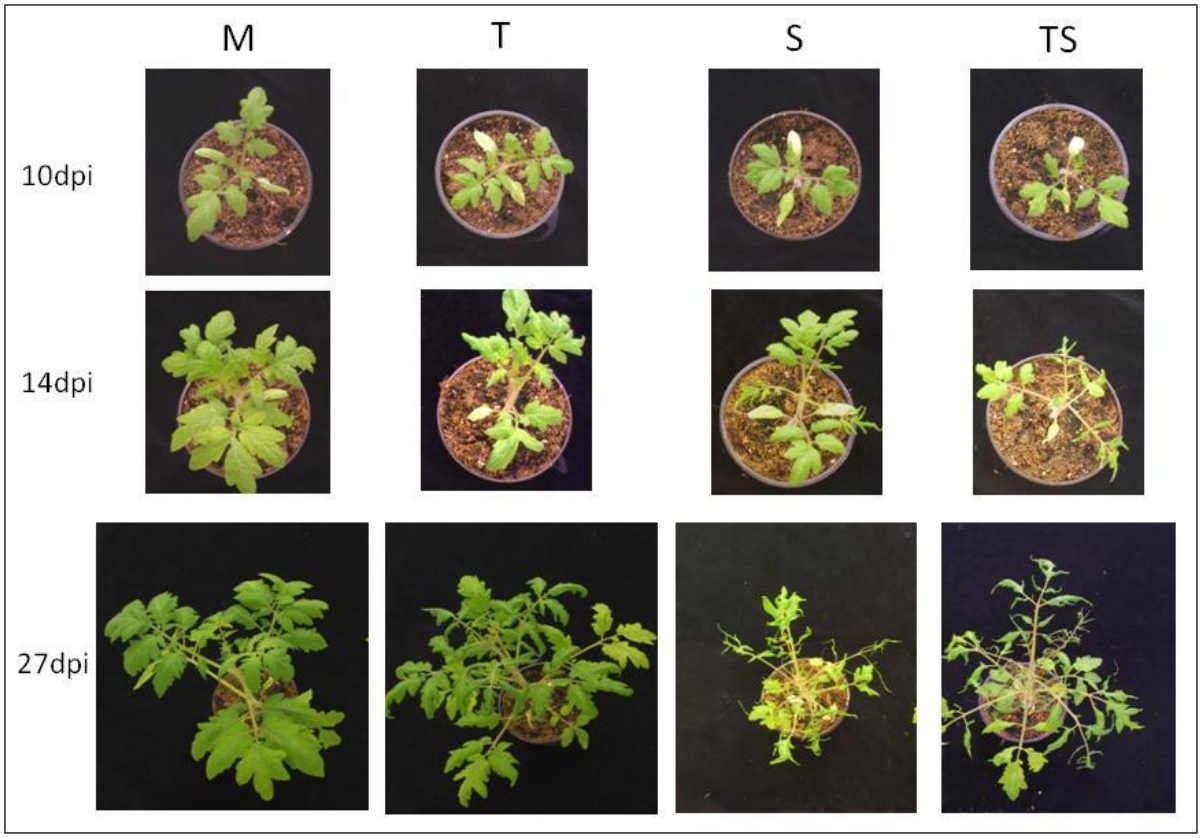


Figure 13. Symptom development in tomato plants at different time points. Mock (M), Trk7-CMV (T), ScL-CMV (S) and Trk7ScL2b-CMV (TS) infected tomato (M82) plants at 10, 14 and 27 days post infection.

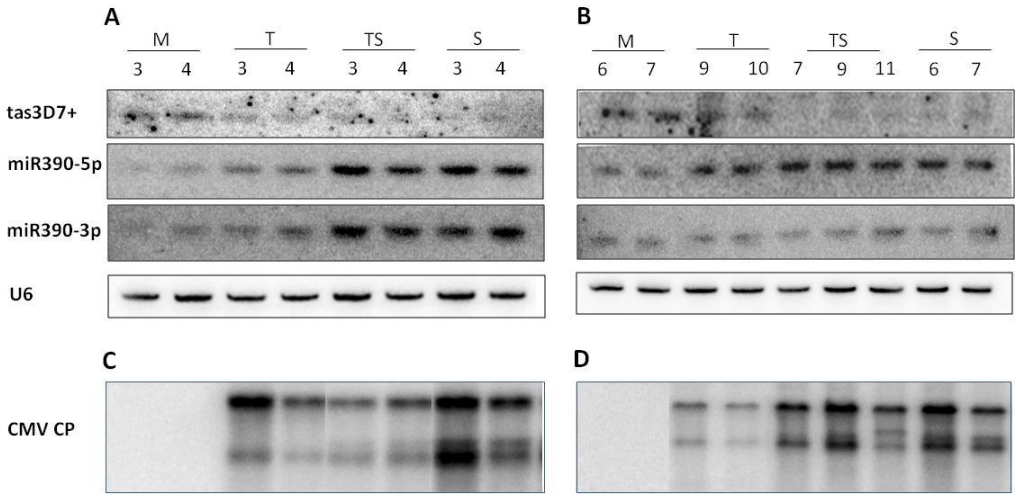


Figure 14. Analysis of different components of tasiR-ARF pathway in CMV infected tomato plants at 14dpi (a) and 27dpi (b). Small RNA northern blot was made from mock (M) Trk7-CMV (T), Trk7ScL2b-CMV (TS) and ScL-CMV (S) infected tomato leaves; numbers means the leaf level (a,b). RNA samples were separated on a 12% polyacrylamide gel. RNA was transferred to a membrane and hybridized with radiolabelled LNA (locked nucleic acid) oligonucleotide (tas3D7+) or with radiolabeled DNA oligonucleotides (miR390-5p and miR390-3p), U6 probes were used as loading controls. The virus level of CMV infected tomato leaves is represented in panel c (14dpi), d (27dpi). Same RNA samples used in small RNA northern blot were separated on denaturing 1% MAE-formaldehyde agarose gel, after transfer to membrane virus level was detected by a DNA probe which was the part of CMV coat protein (CP).

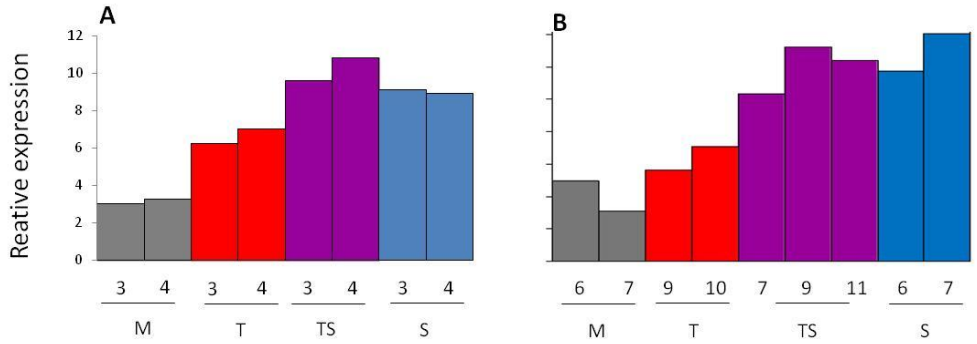


Figure 15. ARF4 gene expression in CMV infected tomato plants at 14dpi (A) and 27dpi (B). qPCR analysis of ARF4 gene expression in mock (M) Trk7-CMV (T), Trk7ScL2b-CMV (TS) and ScL-CMV (S) infected tomato leaves, numbers means the leaf level. Relative transcript levels at different time points were calculated in relation to F-Box gene (*Solanum lycopersicum* F-Box protein NM_001348175).

To summarize our results although ScL-CMV belongs to the subgroup II, causes strong leaf narrowing (shoestring) symptom on tomato plants. We determined that 14. and 35. amino acid of 2b silencing suppressor of ScL-CMV is responsible for the shoestring symptom development. ScL-CMV 2b can not bind siRNA/miRNA and long dsRNA, the other possibility, that it has interaction with a protein component of the plant RNA silencing machinery. In ScL-CMV infected tomato TASS3 derived siRNA important in leaf development (tas3D7+) expression level was decreased and its target gene ARF4 expression was elevated. This elevated expression can cause the leaf narrowing symptom.

Preparation of a manuscript based on these results on “Analysis of molecular background of leaf developmental abnormality caused by virus infection” is already in progress. The results of this work are expected to be a highly cited reference for future studies.

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