UNIVERSITY OF PLOVDIV
"PAISII HILENDARSKI"

FACULTY OF BIOLOGY

'DEPARTMENT OF "PLANT PHYSIOLOGY AND MOLECULAR BIOLOGY'

ABSTRACT

of a dissertation to acquire the
the educational and scientific degree "doctor"

"Identification of specific interactions between Potato Spindle Tuber Viroid and two Bulgarian pepper cultivars"

by

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Field of higher education: 4. Natural sciences, mathematics and informatics
Professional direction: 4.3. Biological Sciences
PhD program: "Molecular Biology"

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Plovdiv, 2023

The dissertation contains 121 pages, 7 tables, 33 figures and 240 references.

The PhD student has published a total of 3 articles in journals with an impact factor. The dissertation is based on two of these articles and 1 in a journal with no impact factor.

The dissertation work was discussed and proposed for defense to the departmental council of the Department of "Plant Physiology and Molecular Biology" at the Faculty of Biology of the PU "P. Hilendarski", held on February 6, 2023.

The open final meeting of the scientific jury will be held on 12/05/2023 at 12:00 in the Faculty of Biology (2 Todor Samodumov Street, Plovdiv).

The materials on the defense are provided for free access of your interests in the library of PU "Paisiy Hilendarski".

Author: Nikol Slaveva Hadjieva

Title: Identification of specific interactions between Potato Spindle Tuber Viroid and two Bulgarian pepper cultivars

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ABSTRACT

Plants are subject to the action of various environmental factors. Abiotic factors include radiation, salinization, flooding, drought, extreme temperatures, heavy metals, and biotic factors include viruses, viroids, bacteria, fungi, insects, nematodes, and herbivores. When multiple factors interact with a single plant, it can lead to antagonistic or synergistic effects. Plant responses to different types of stress are very complex and involve changes at the transcriptomic, cellular and physiological levels. Understanding the multi-layered regulation that plants use to manage relationships with their biotic environment is fundamental to developing stress-tolerant crop plants. RNA silencing operates as part of a complex network of interconnected pathways for cellular defense, RNA control and plants development. This mechanism represents a unique type of gene regulation based on RNA target specificity. By means of short regulatory RNA molecules, it modulates gene expression and thus affects the immunity of the plant.

Viroids are the smallest RNA plant pathogens. The infections they cause on cultivated species lead to serious economic losses related to a reduction in their yield or loss of the entire crop. In the defense response to viroid infection, plants involve different pathways and regulatory networks. Endogenously expressed micro RNA molecules are a subtle mechanism to regulate the expression levels of specific mRNAs through destabilization or translational inhibition. Knowledge about viroid- induced changes in microRNAs levels, and their regulated target genes, would contribute to the development of a more effective strategies of protection against viroid infections.

INTRODUCTION

The introduction describes viroids as infectious agents, their life cycle and their interactions with hosts. The main highlights are:

1. Potato Spindle Tuber Viroid (PSTVd), the causative agent of spindle tuber on potato, as an infectious agent on members of the *Solanaceae* family
2. Viroid-induced RNA silencing as an antiviral defense mechanism; characterization and biogenesis of small interfering RNAs
3. Biogenesis and role of microRNAs in the PSTVd-specific response
4. Large-scale expression analysis of various pathosystems involving pepper

AIM AND TASKS

The **aim** of the present work is to identify specific interactions between Potato Spindle Tuber Viroid and two Bulgarian pepper cultivars.

The following tasks were set:

1. Conducting bioassays with PSTVd *in vitro* transcript on Bulgarian pepper varieties 'Kurtovska kapia' and 'Djulyunska shipka', which showed PSTVd - specific phenotype.
2. Isolation of total RNA from leaf samples of mock and PSTVd-infected plants of both cultivars.
3. Next-generation sequencing of small RNA and mRNA libraries prepared from mock and PSTVd- infected plants of both cultivars.
4. Conducting bioinformatic sequencing data analysis to detect differentially expressed microRNA genes and protein-coding genes.
5. Conducting expression analysis by RT-qPCR of the selected differentially expressed protein-coding genes and microRNA genes.
6. Conducting a gene-ontology analysis of the differentially expressed protein-coding genes.

MATERIALS AND METHODS

1. Materials

1.1. Plant material

For the purposes of the experimental work, the Bulgarian pepper cultivars - Djulyunska shipka (DS) and Kurtovska capia (KK) (Capsicum annuum L.) were selected, of which DS showed the most pronounced PSTVd phenotype such as leaf curling and malformations in the conducted preliminary bioassay.

1.2 PSTVd viroid isolate

The PSTVd viroid isolate KF440-2 was used for the purpose of the present study.

2. Methods

1. Bioassays on selected Bulgarian pepper cultivars with *in vitro* synthesized PSTVd (+) RNA
2. Isolation of total RNA from leaf samples of KK and DS cultivars at 28 and 43 dpi
3. Quantitative and qualitative analysis of total RNA by spectrophotometric measurement and electrophoretic separation on agarose gel
4. Next-generation sequencing of small RNAs and mRNAs

4.1 Next-Generation Sequencing of Small RNAs by Macrogen Inc. Korea.

Two libraries (mock and infected) for each cultivar (KK and DS) were prepared and sequenced. Single-end runs of 50 nt length were sequenced.

4.2 Next-generation analysis of mRNA on the Illumina HiSeq 2000 platform, conducted by Novogene Technology Co., Ltd (Beijing, China).

Four libraries of mock and infected samples for each cultivar (KK and DS) were prepared and sequenced. 150 nt paired ends sequencing reads were generated.

5. Synthesis of complementary DNA
6. Standard polymerase chain reaction (PCR) used for specific amplification of PSTVd or pepper genes
7. Quantitative analysis of relative gene expression

In the present study, RT-qPCR was performed with the fluorescent dye SYBR Green I. The constitutive genes: EF1α and U6 were used for expression normalization. Evaluation of the expression of the studied genes was carried out using the "$2^{-\Delta\Delta Ct}$" method described by Livak and Schmittgen.

7.1 Analysis of miRNA expression levels by RT-qPCR

Stem-loop and forward/reverse primers were designed for: can-miR482a-3p, stu-miR6024- 3p, can-miR398c-3p, can-miR408a, can-miR408-3p, can-miR397a-5p, and can-miR397b, according to the work of Chen and co-workers (2005).

7.2 Analysis of expression levels of protein-coding genes by RT-qPCR

Primer pairs were designed for the following six protein-coding genes: CA02g15240 (auxin-repressed 12.5 kDa protein-like isoform), CA10g00480 (NADPH: protochlorophyllide oxidoreductase [POR]), CA07g02110 (polygalacturonase-inhibiting protein [PGIP]), CA07g11190 (1 -aminocyclopropane-1-carboxylic acid oxidase [ACO]), CA02g26610 (S-adenosylmethionine decarboxylase proenzyme [SAMDC]), and CA09g02410 (phenylalanine ammonia-lyase [PAL]).

8. Bioinformatics analyses

8.1 Next-generation sequencing of small RNAs and mRNAs

The next-generation sequencing reads were processed in-house workflow in the Galaxy platform (FastQC (Galaxy version 0.73) and TrimGalore (Galaxy version 0.6.7).

PSTVd specific small RNAs were identified by mapping sequencing reads to PSTVd KF440-2 genome.

The identification of conserved miRNAs was accomplished by comparing the sequencing reads with a local database that contains *Solanaceae* miRNAs from miRBase 21.0 and the published pepper miRNAs by Hwang et al, 2013 and Qin et al 2014.

Differential gene expression analysis was based on DESeq2 package (Galaxy version 2.11.40). The clean reads from mRNA sequencing were mapped to the Zunla 1 v.2.0 reference genome on a Galaxy platform via HISAT2 (Galaxy version 2.2.1). The number of gene transcripts was determined according to the annotated transcripts for pepper. DEGs with $|\log2FC| \geq 1$ and $p < 0.05$ were selected for further analysis.

GO enrichment analysis was performed using g:Profiler for the selected genes. The tool is available on server g:GOSt with g:SCS- multiple testing correction method using gSCS significance threshold (0.05).

Data from next-generation sequencing of small RNAs have been deposited in the NCBI Sequence Read Archive(SRA) database: (https://dataview.ncbi.nlm.nih.gov/object/PRJNA631129?reviewer=l0686rrde7fsor65p7j623vad), while the next-generation mRNA sequencing data have been deposited under the accession number: PRJNA762255 (https://www.ncbi.nlm.nih.gov/sra/PRJNA762255).

RESULTS

1. Bioassays with PSTVd on pepper cultivars

1.1 Synthesis of *in vitro* PSTVd transcript

The PSTVd *in vitro* transcript was synthesized using plasmid pH106 according to the protocol described in detail in M&M. Agarose gel electrophoresis was performed to verify the quantity and

quality of the *in vitro* PSTVd transcript (Fig.1). The analysis showed that the obtained transcript was of good quality and could be used to conduct the bioassays.

1.2 Mechanical inoculation of PSTVd on two Bulgarian pepper cultivars

Bioassays were conducted on the selected cultivars Kurtovska kapia (KK) and Djulynska shipka (DS) and infection was monitored at 14, 28 and 43 days post inoculation (dpi), similar to the periods at which PSTVd infection was investigated in tomato.

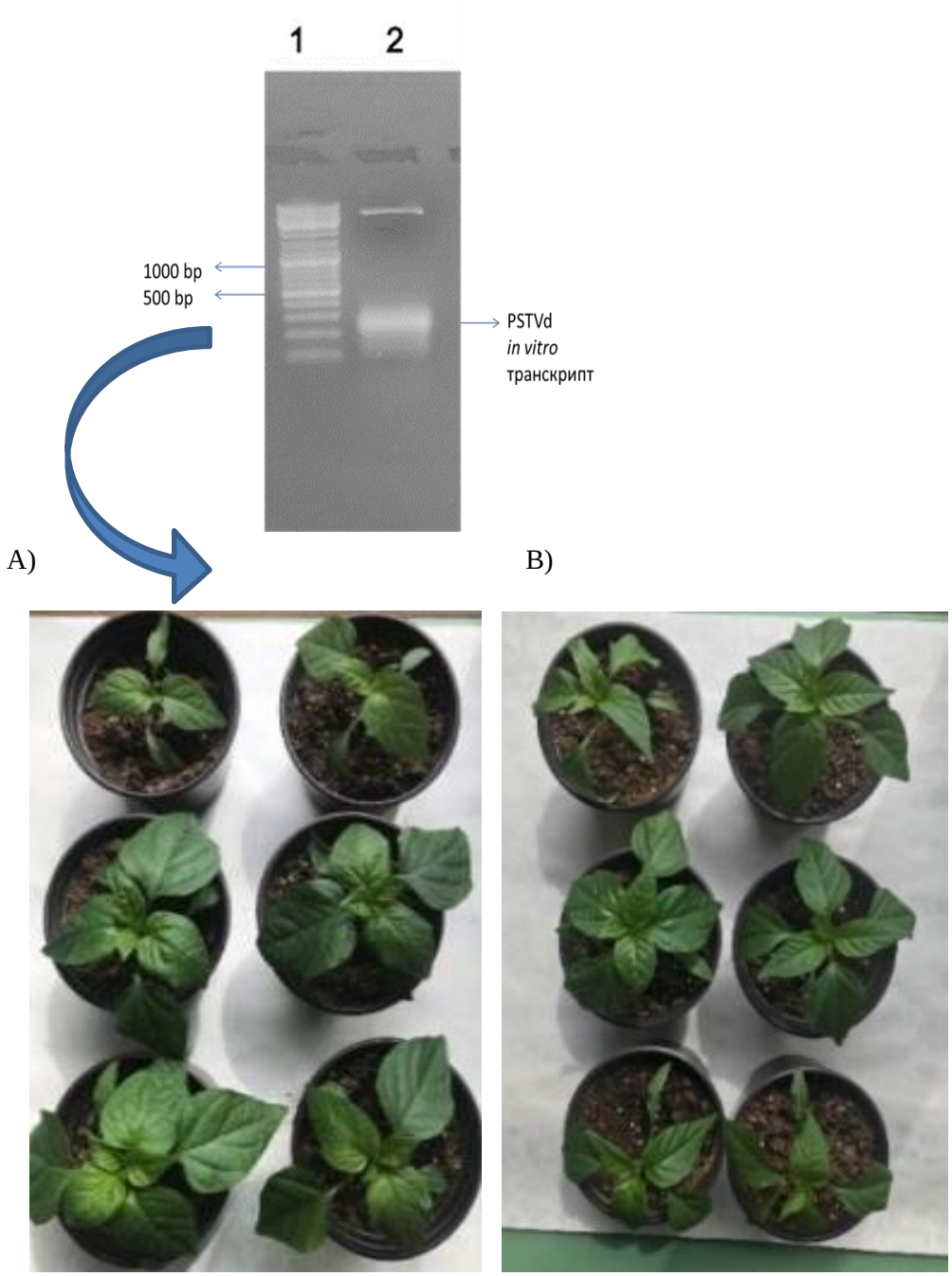


***Fig. 1**: PSTVd inoculation of pepper cultivar KK (A); pepper cultivar DS (B) at the second true leaf stage. PSTVd in vitro transcript separated on agarose gel electrophoresis is presented in the upper part of the figure.*

The development of PSTVd specific symptoms in both cultivars at 28 and 43 dpi is presented in Fig. 2

A) B)

***Fig. 2**: Comparative phenotypic analysis between mock and PSTVd-infected plants DS (A) and KK (B) at stage 28 dpi and 43 dpi. Mock plants are presented in the first row and infected plant in the second row.*

2. Analysis of the quantity and quality of isolated total RNA from KK and DS

Leaves from five individual plants from each experimental group (mock and infected plants of both cultivars) were collected and pooled for total RNA extraction. Isolation of RNA from pepper is difficult because of polysaccharides and polyphenols. To overcome these limitations, plants were darkened from the previous day. Isolation of RNA was performed with Qiazol lysis reagent and subsequent purification on a Nucleospin miRNA kit column (Masherey-Nagel). Total RNA was isolated and analyzed quantitatively and qualitatively at both 28 and 43dpi.

RNA was analyzed using a Bioanalyzer 2100 (Agilent) and its integrity was characterized. An electropherogram and a gel-like image were generated showing the concentration of RNA and the ratio between the 18S and 25S rRNA. A RIN software algorithm was applied, which ranks the integrity of eukaryotic total RNA on a scale of 1 to 10, with 1 is RNA with the most degraded profile and 10 being the most intact profile. The results of the analysis of the RNA samples with the Bioanalyzer 2100 show RIN values >6, which corresponds to the high quality of the isolated RNA, which is an important issue

for obtaining reliable data on gene expression. The data obtained by the Bioanalyzer 2100 were confirmed by agarose gel electrophoresis (Fig. 3).

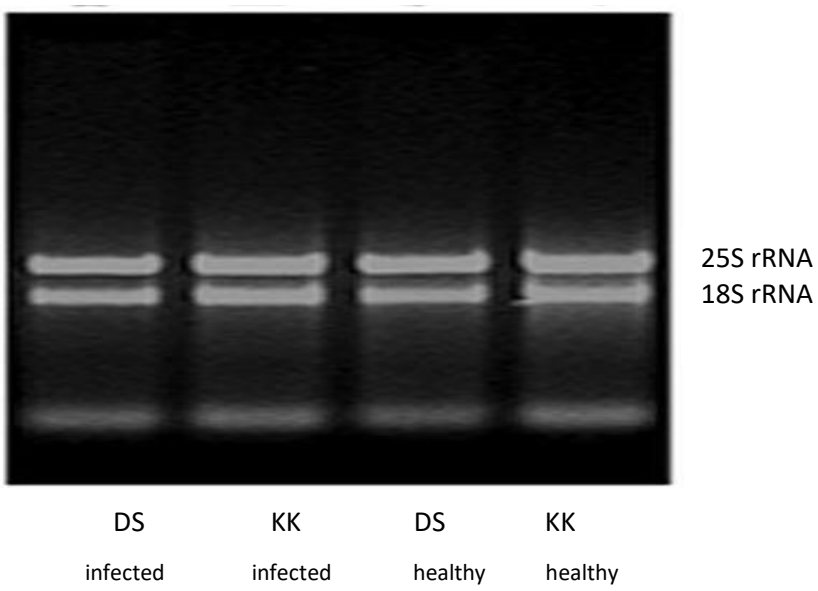


***Fig. 3**: Electrophoretic profile of total RNA enriched in small RNA, on a 1% agarose gel, isolated from mock and PSTVd infected DS and KK at 43 dpi. RNA is a pool of at least three individual plants.*

3. PCR analysis to detect PSTVd (+) RNA in KK and DS

Complementary DNA obtained with PSTVd Rev primer was used as a template in conventional PCR with PSTVd-specific primers. A specific 360 bp amplicon corresponding to the PSTVd (+) chain was found to be present in PSTVd-inoculated plants of both pepper cultivars. The obtained results prove a successful PSTVd infection (Fig. 4).

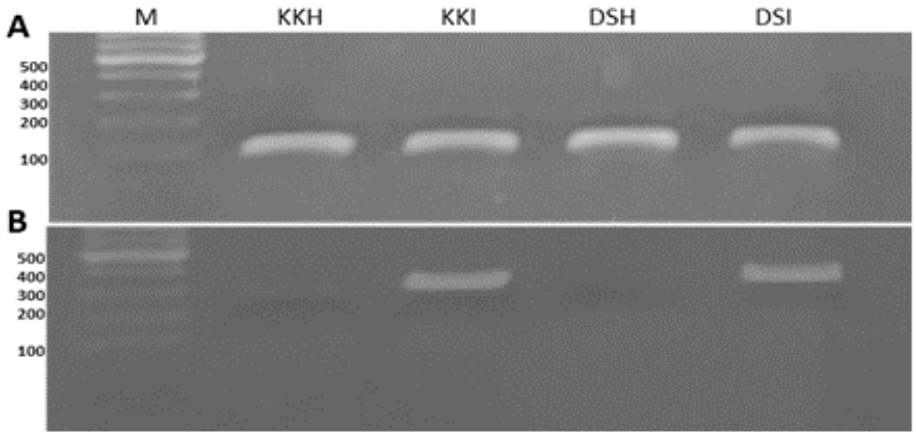


***Fig. 4**: Detection of PSTVd (+) chain in mock and infected KK and DH plants by RT-PCR (B), the constitutively expressed gene EF1a (A) was used as a positive control.*

4. Analysis of relative accumulation of PSTVd in the two pepper cultivars

Semi-quantitative RT-PCR was used to study the PSTVd titer in both cultivars. The reaction was carried out under non-saturating PCR conditions (28 cycles) using complementary DNA (diluted 2, 3 and 4 times) and primers for amplification of the PSTVd (+) strand and the reference gene EF1a. The results of the performed RT-PCR showed a slightly higher accumulation of PSTVd in the infected DS compared to KK, that mightly be related to the stronger phenotype manifested by DS (Fig. 5).

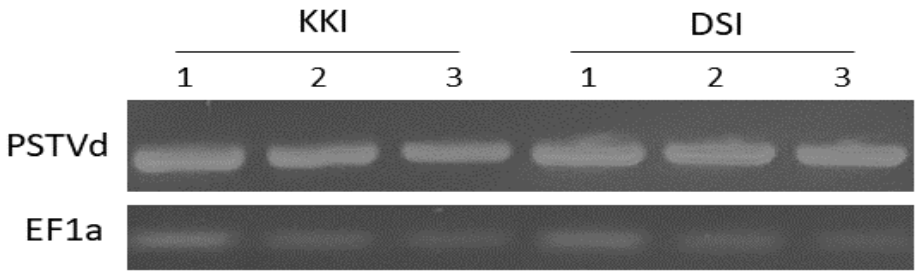


Fig. 5*: Semi-quantitative RT-PCR analysis for the detection of the relative accumulation levels of the PSTVd (+) chain in infected KK and DS.*

5. Analysis of next-generation sequencing of small RNAs

Next-generation sequencing of small RNAs was performed to study the profile of these regulatory RNAs in response to PSTVd infection in pepper. The analysis was performed at the 28 dpi, when the molecular changes leading to the development of mild symptoms visible during the late phase of infection (43 dpi) are supposed to take place. The resulting raw data were processed to remove adapters, filter reads corresponding to ribosomal RNAs, remove reads with unknown bases (Ns). 62,078,305 clean reads were obtained for KKH; 59,524,663 for KKI; 51,075,609 for DSH; and 49,176,658 for DSI.

Analysis of the obtained small RNA libraries of both mock and infected plants showed that the size of the small RNAs in the libraries ranged from 18 to 30 nt, and a major part of them were 21–24 nt in length (Fig. 6).

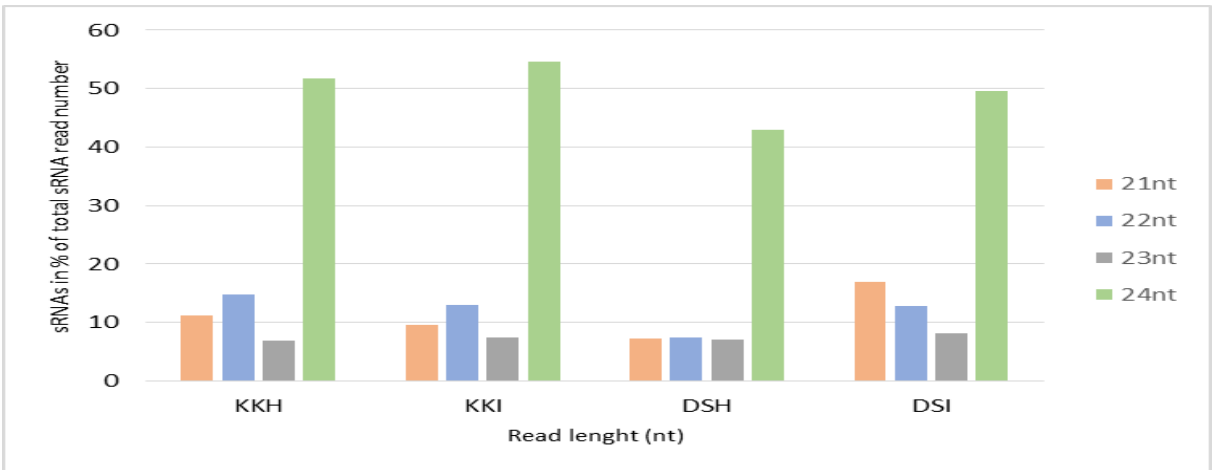


Fig. 6*: Distribution of small RNAs by size in mock and infected samples of KK and DS at stage 28 dpi. Each class of small RNAs is represented as a % of the total number of small RNA reads.*

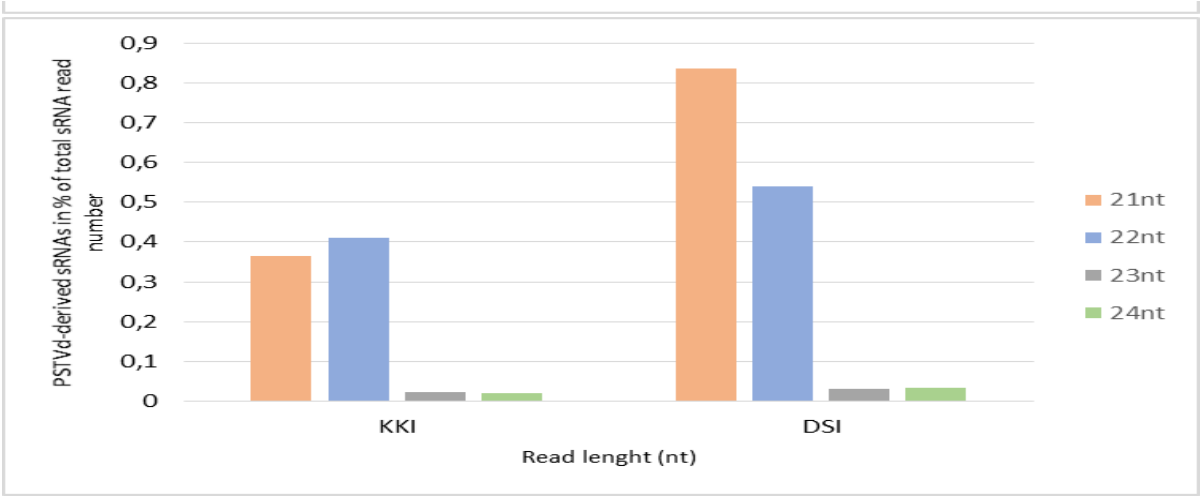
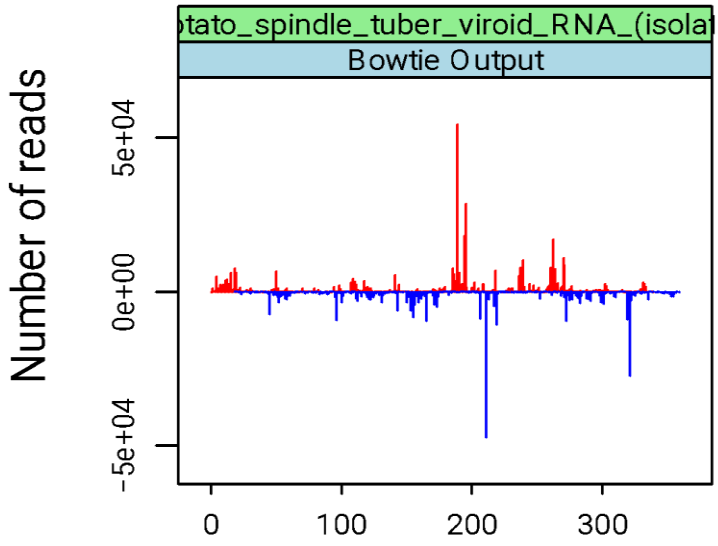


Fig. 7: *Size distribution of 21, 22, 23, and 24 nt PSTVd-specific RNAs in the analyzed DSI and KKI libraries at 28dpi. Each class of PSTVd-specific RNAs is represented as a % of the total number of small RNA reads.*

The predominant size class small RNAs in all libraries was found to be 24 nt. Analysis of the PSTVd-specific RNAs detected in the DSI and KKI libraries showed that they represented a small percentage of the total reads, but originated from both PSTVd strands. In DSI, PSTVd-specific small RNAs with a length of 21 nt were 0.83% of the total number of reads, while in KKI they constituted only 0.36%. The class of 22 nt small RNAs were equally represented in DSI (0.54%) and KKI (0.41%) (Fig. 7).

5.1 Mapping small RNAs onto a PSTVd reference genome

Mapping of the small RNAs from libraries of the infected KK and DS was carried out on the reference PSTVd KF440-2 genome (Fig. 8).



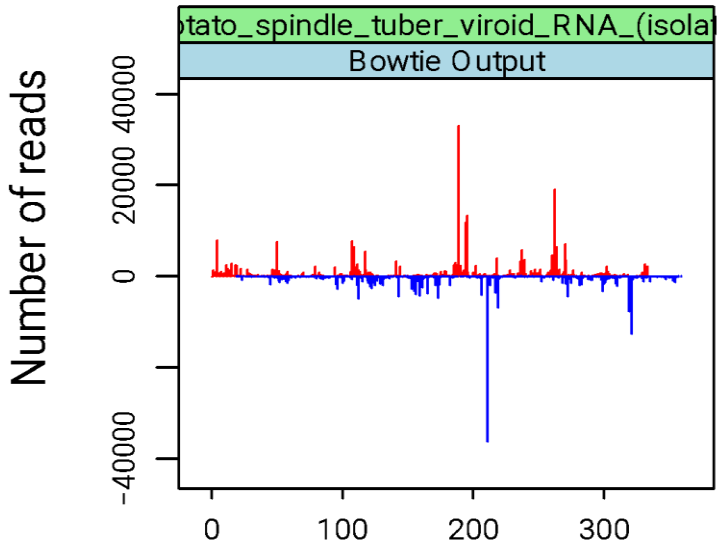


***Fig. 8**: Plot diagram showing the amount of mapped sequences on the reference genome of PSTVd (KF440-2) for cultivar KK (A) and DS (B).*

The analysis showed a distribution of reads over the entire length of the (+) and (-) strands of PSTVd. The numbers of generated PSTVd-specific small RNAs from both strands were comparable, indicating that the viroid replicates in plants and both strands are accessible to the RNA silencing enzyme system.

Peak plot analysis showed that certain positions of the PSTVd genome produced significantly more PSTVd-specific small RNAs than others (Fig. 8). When comparing the plot diagrams generated for the two cultivars KK and DS, no differences related to cultivar-specific response were found. The highest production of PSTVd-small RNAs in both cultivars was found in the 180-220nt and 250-270nt regions, which cover the terminal right and left domains of PSTVd (Fig. 8). This fact suggests that this part of the viroid genome is more accessible to host nucleases.

5.2 Conserved miRNAs and their families altered in response to PSTVd infection

MiRNAs identified from all four libraries of infected and mock KK and DS were classified into corresponding miRNA families. In the KKH sample, 217 miRNAs belonging to 71 families were detected; in KKI, 210 miRNAs belonging to 69 families; DSI, 209 miRNAs belonging to 66 families; 217 miRNAs from 66 families (Fig. 9). The miR166, miR169, and miR395 families were found to be present with more members in the DS libraries compared to the KK libraries. More miR156 and miR399 members were predominant in the infected libraries compared to the mock libraries in both cultivars (Fig. 9).

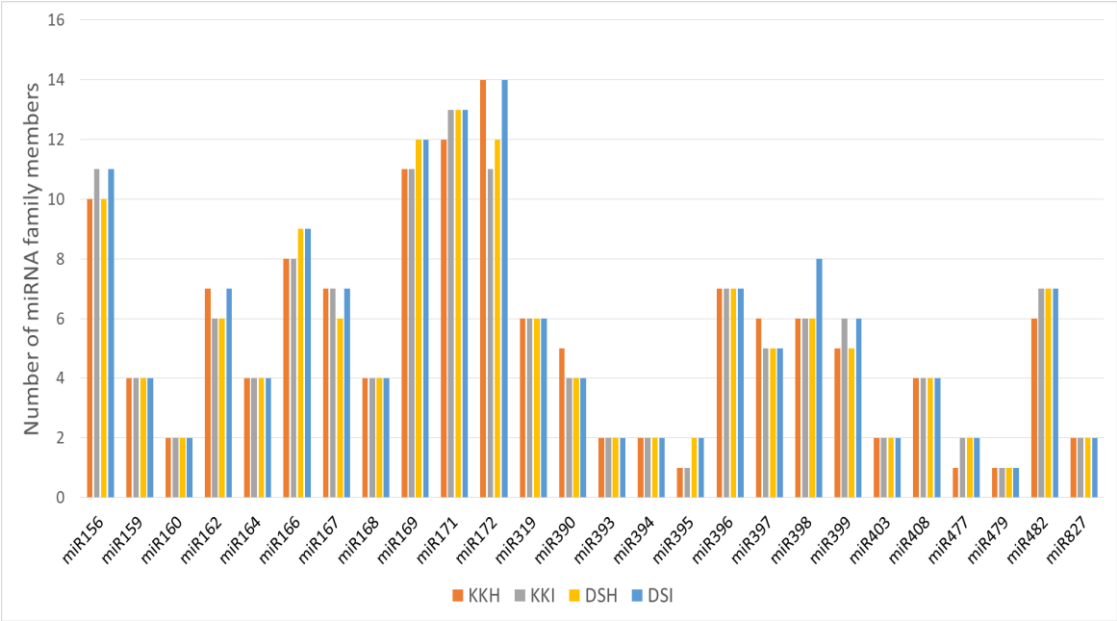


***Fig. 9**: Conserved miRNAs and their families identified in the four small RNA libraries from KK and DS.*

5.3 MicroRNA genes with opposite expression profiles in response to PSTVd infection

Changes in miRNA expression in PSTVd-infected plants at 28 dpi compared to mock plants were calculated for each cultivar using the DEseq2 package (Love et al., 2014) (Fig. 10). 14 differentially expressed miRNAs were identified in KK and, respectively, 32 in DS with $|\log2FC| \geq 1$ (Fig. 10).

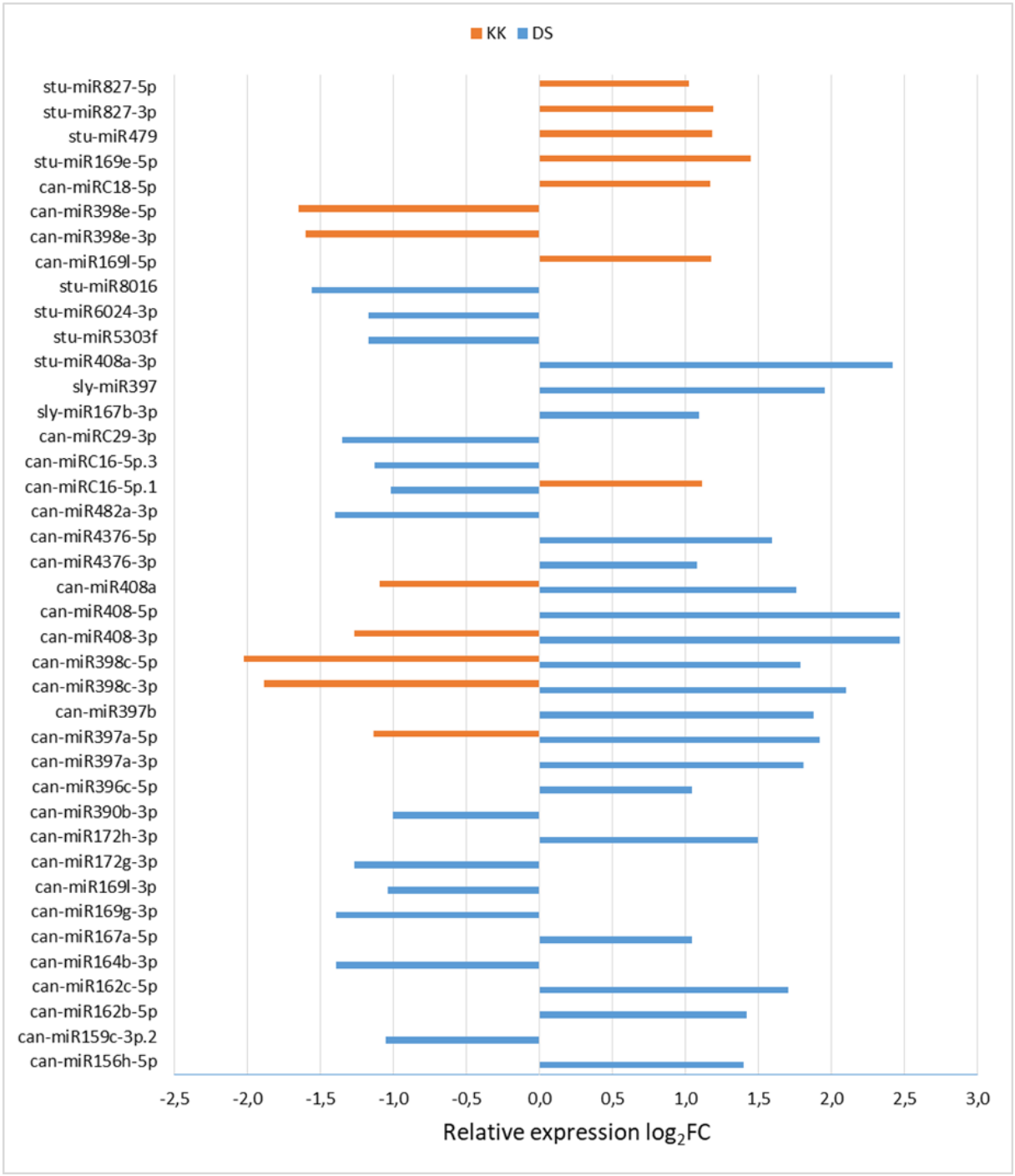


***Fig. 10**: Differentially expressed microRNAs with |log2FC| ≥ 1 and p < 0.05 between KKI and KKH, and between DSI and DSH at stage 28 dpi.*

Among the differentially expressed miRNAs in KK in responswe to PSTVd, the number of miRNAs with increased expression was comparable with that of miRNAs showing decreased expression (Fig. 10). The greatest reduction in expression was observed in miRNAs belonging to the miRNA398 family (miR-398c-5p, miR-398c-3p, and miR-398e-3p), as well as in can-miR408-3p and can-miR397a-5p. A significant increase in expression levels was reported for stu-miR169e-5p, a slight increase in expression was also found for two pepper-specific miRNAs (miRC16 and miRC18) (Fig. 10).

In PSTVd-infected DS plants, a significant number of miRNAs with increased expression (19 of 32) were observed (Fig. 10). These miRNAs include several members of the miR408 family (can-miR408-3p, can-miR408a, and stu-miR408a-3p) and the miR397 family (can-miR397b, can-miR397a-5p, and sly-miR397). miRNAs showing decreased expression in DS were can-miR482a-3p, can-miR164b-3p, can-miR172g-3p, and stu-miR6024-3p. The pepper-specific miRNA, miRC16, also had a slight down-regulation of expression in DS.

5.4 Validation of selected miRNA genes that showed an opposite expression profile between the two cultivars

From the differentially expressed miRNAs with a read count ≥ 100 and showing an opposite profile between the two cultivars, seven miRNAs were selected for validation by RT-qPCR. Table 1 presents the nucleotide sequences of these miRNAs and their |log2FC| expression ≥ 1, according to the next-generation sequencing of small RNAs.

miRNA	miRNA nucleotide sequencing	cultivar	Read Number	Log2 FC
can-miR408a	TGCACTGCCTCTTCCCTGGCT	DS	20837.23	1.75
		KK	10418.59	-1.09
can-miR408-3p	TGCACAGCCTCTTCCCTGGCT	DS	4818.88	2.46
		KK	2004.02	-1.26
can-miR398c-3p	TGTGTTCTCAGGTCGCCCCTG	DS	22962.56	2.10
		KK	20220.60	-1.88
can-miR397a-5p	TCATTGAGTGCAGCGTTGATG	DS	3598.06	1.91
		KK	2378.80	-1.13
stu-miR6024-3p	TTTTAGCAAGAGTTGTTTTCCC	DS	3953.11	-1.17
can-miR397b	ATTGAGTGCAGCGTTGATGAC	DS	5437.95	1.87
can-miR482a-3p	TTTCCAATTCCACCCATTCCTA	DS	2846.59	-1.40

Table 1: *List of differentially expressed miRNAs with an opposite profile between the two cultivars selected for validation by RT-qPCR*

The RT-qPCR results show cultivar-specific dynamics and confirm the data from next-generation sequencing of small RNAs. In response to PSTVd infection, can-miR398c-3p, can-miR408a can-miR408-3p, can-miR397b, and can-miR397a-5p had increased expression in DS and decreased in KK

(Fig. 11), while can-miR482a -3p shows a reduction in expression in DS and an increase in expression in KK. The only miRNA for which cultivar-specific expression was not confirmed was stu-miR6024-3p. A reduced expression was reported for it in both cultivars.

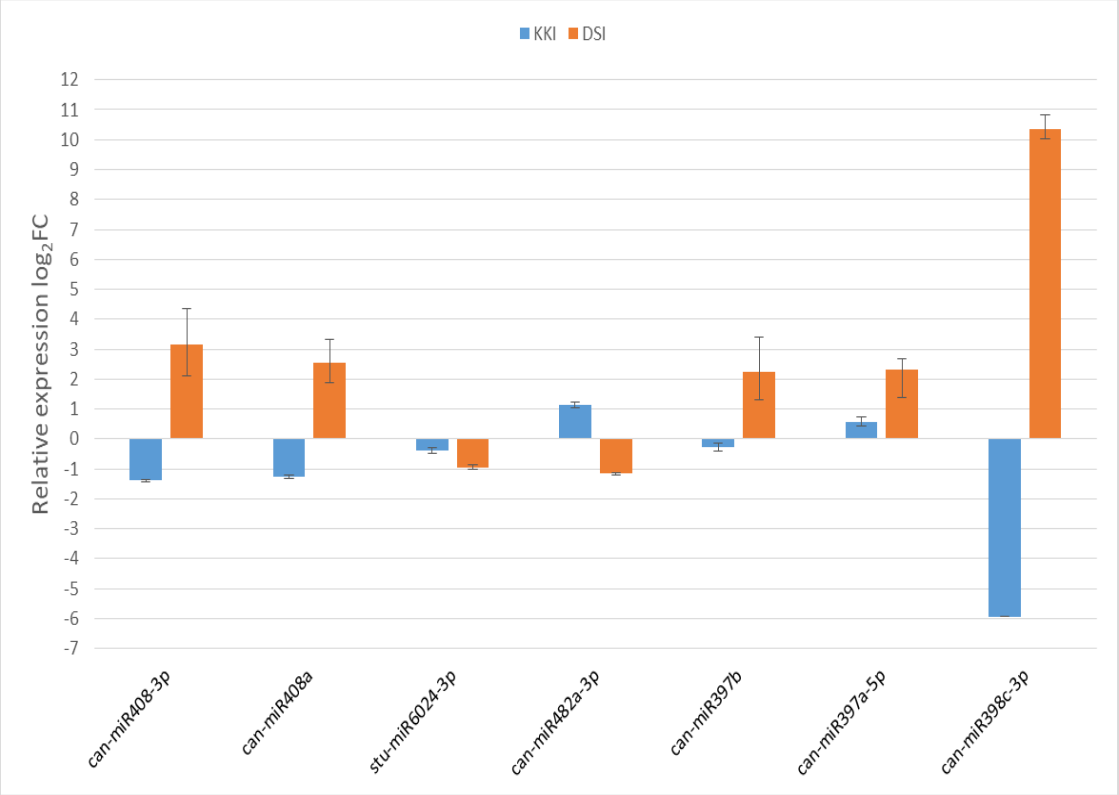


***Fig. 11**: Expression analysis of can-miR408-3p, can-miR408a, stu-miR6024-3p, can-miR482a-3p, can-miR397b, can-397a-5p, and can-miR398c-3p by RT-qPCR in KK and DS at 28dpi.*

6. Analysis of Next-generation sequencing of mRNA

In connection with the next-generation sequencing of mRNA, four libraries were created: DS mock; DS infected; KK; KK infected. Next generation mRNA sequencing generated over 173 M reads.

6.1 Differentially expressed protein-coding genes in response to PSTVd infection in KK and DS

For pairwise comparative expression analysis between mock and PSTVd-infected plants, the DESeq2 method was used. The analysis identified 998 differentially expressed genes (DEGs) in KK plants, of which 43.5% were down-regulated and 56.5% up-regulated, and 2161 DEGs in DS plants, of which 62.3% were down-regulated and 37.7% up-regulated (Fig. 12).

Fig. 12: *Genes with increased and decreased expression in pepper cultivars KK and DS in response to PSTVd infection at 43 dpi.*

The Venn diagram of DEGs in both cultivars shows that the number of DS-specific DEGs is twice as high as KK-specific DEGs in response to viroid infection (Fig. 13).

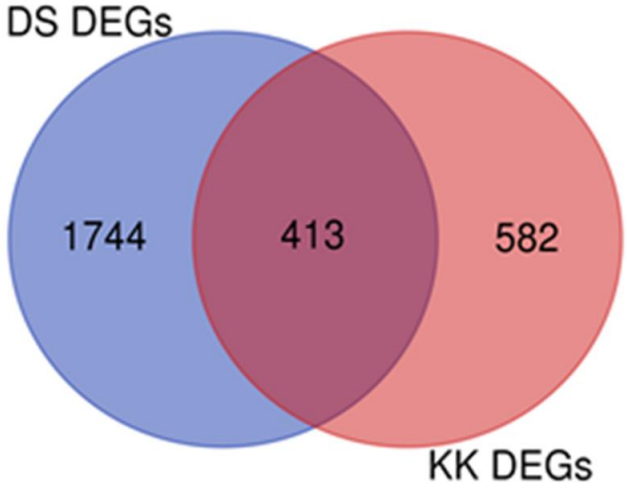


Fig. 13*: Venn diagram showing common and specific DEGs for KK and DS in response to PSTVd infection 43dpi.*

Among DEG in DS, 1346 genes were down-regulated and 564 genes were up-regulated in DS. In KK, 434 genes were down-regulated and 815 genes were up-regulated. 413 of the genes identified in both cultivars were overlapping DEGs (Fig. 13).

6.2 DEGs exhibiting opposite expression patterns in KK and DS in response to PSTVd infection

Analysis of 413 total DEGs showed that 224 DEGs exhibited opposite expression patterns in the two cultivars. Some of these DEGs with the opposite expression profile are presented in Fig. 14.

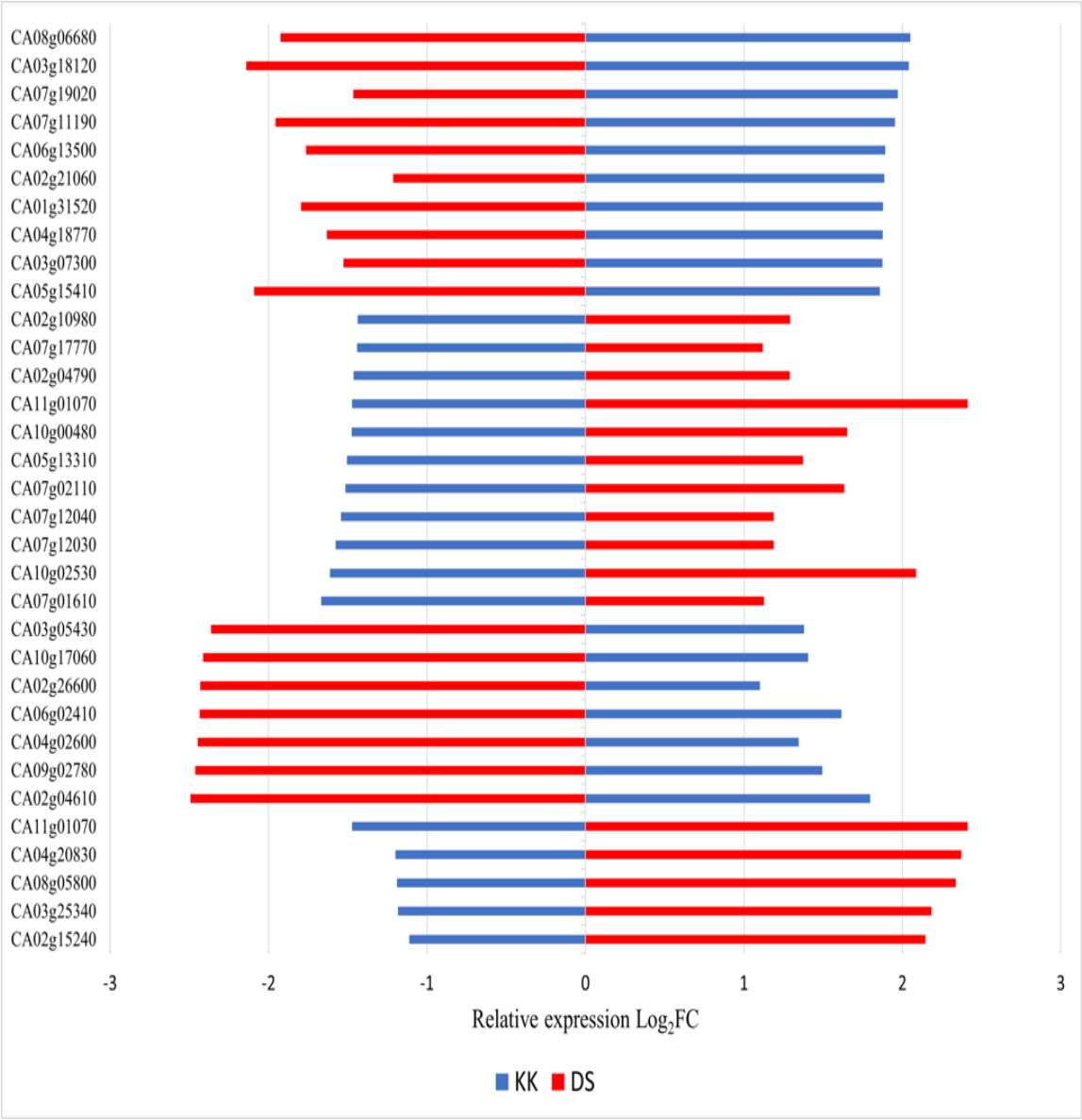


Fig. 14*: DEGs with |log2FC| ≥ 1 exhibiting opposite expression patterns in the two pepper cultivars in response to PSTVd infection, according to next-generation mRNA sequencing data.*

6. 3 Differentially expressed transcription factors identified in the two cultivars in response to PSTVd infection

Among the overlapping DEGs, 23 transcription factors (TFs) were identified (Fig. 15), the majority of which showed a change in expression in the opposite direction (Table 2).

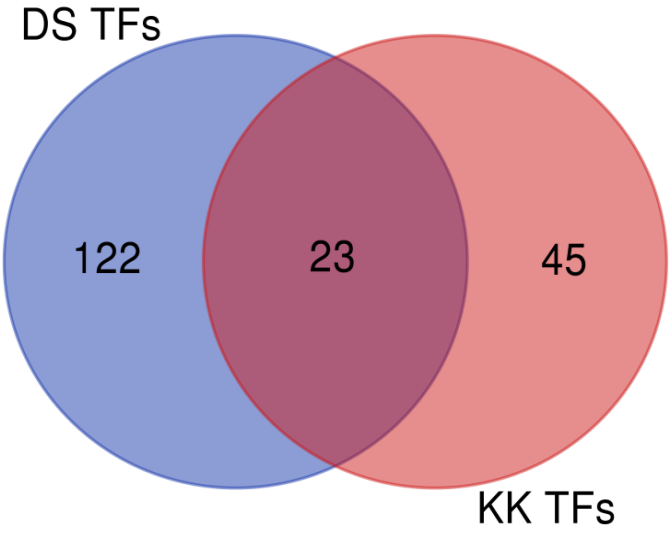


Fig. 15 : *Venn diagram representing the number of DE TFs (TFs were extracted from PlantRegMap/PlantTFDB v5.0).*

Accession number	Read Number in DS	Log2FC DH	Read Number in KK	Log2FC KK	Gene name
CA02g26670	3311.00	-2.30	5504.44	-1.31	CONSTANS protein
CA02g07170	223.52	-2.27	1791.25	-1.51	Myb-like DNA-binding protein%2C putative
CA08g16870	458.66	-2.25	165.18	1.03	Zinc-finger DNA binding protein
CA02g22440	331.70	-2.22	76.41	-1.42	protein RADIALIS-like 1-like [Glycine max]
CA08g08650	620.32	-2.15	308.82	1.58	homeobox-leucine zipper protein ATHB-7-like isoform 1 [Solanum lycopersicum]
CA09g11950	128.57	-1.97	82.94	1.04	NtWRKY1
CA02g07270	83.25	-1.91	61.83	1.41	Homeodomain leucine-zipper 1
CA10g02900	175.04	-1.91	165.96	-1.15	Golden 2-like 2 transcription factor
CA02g01800	349.24	-1.68	312.75	1.06	WRKY transcription factor 26
CA07g21470	137.43	-1.61	70.39	-1.12	Salicylic acid-induced protein 19
CA05g04410	1005.68	-1.59	967.21	1.28	NAC transcription factor
CA10g20490	29.72	-1.51	105.26	1.22	CCAAT-binding transcription factor
CA03g36090	96.11	-1.36	424.16	-1.54	DNA binding protein%2C putative
CA02g30960	15.09	-1.24	69.61	1.45	probable WRKY transcription factor 45-like [Solanum tuberosum]
CA03g06220	195.40	-1.15	131.81	1.21	Basic helix-loop-helix protein
CA10g20440	235.88	-1.12	267.01	1.29	Heat shock factor protein%2C putative
CA04g17180	88.32	1.08	52.09	-1.11	Transcription factor bHLH
CA02g26690	390.86	1.09	28.32	-1.21	CONSTANS protein
CA10g06890	20.48	1.25	65.22	1.44	WRKY transcription factor 6
CA11g16170	998.41	1.40	586.89	1.06	BEL5 protein
CA04g16680	36.63	1.45	88.77	1.41	R2R3-MYB transcription factor MYB1.1

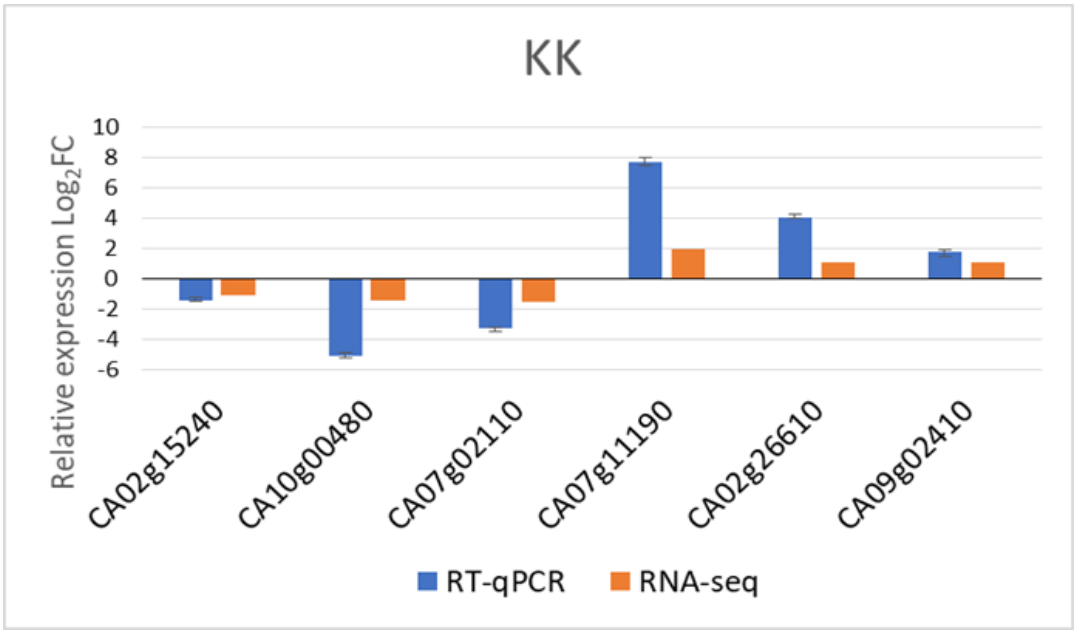
CA04g19220	132.97	2.22	44.93	1.15	Tuber-specific and sucrose-responsive element binding factor
CA11g01070	1508.63	2.41	178.09	-1.47	zinc finger protein CONSTANS-LIKE 16-like [Solanum lycopersicum]

***Table 2**. DE TFs in KK and DS described with their SOL Genomics Network database accession number, number of reads, and expression, represented as |log2FC| ≥ 1 in response to PSTVd.*

The majority of DS-specific TFs are down-regulated, while most of the KK-specific TFs are up-regulated (information is presented in supplementary tables attached to the electronic version of the dissertation).

6.4 Comparative expression analysis of selected overlapping DEGs in response to PSTVd infection

Among all overlapping DEGs showing opposite expression patterns in the two cultivars, six genes were selected for validation by quantitative RT-qPCR. Among these six genes, CA02g15240 (auxin-repressed 12.5 kDa protein-like isoform), CA10g00480 (NADPH: protochlorophyllide oxidoreductase [POR]), and CA07g02110 (polygalacturonase-inhibiting protein [PGIP]), showed decreased expression in infected KK and increased expression in infected DS plants, while CA07g11190 (1-aminocyclopropane-1-carboxylic acid oxidase [ACO]), CA02g26610 (S-adenosylmethionine decarboxylase proenzyme [SAMDC]), and CA09g02410 (phenylalanine ammonia-lyase [PAL]) showed increased expression in KK and decreased in DS as a result of infection, according to data from next-generation mRNA sequencing. The RT-qPCR analysis confirmed the gene expression profiles in the infected KK and showed that CA10g00480, CA02g26610 and CA07g11190 underwent the most significant change in expression. In the infected plants of cultivar DS, two genes (CA10g00480 and CA07g11190) showed differences compared to next-generation mRNA-sequencing data (Fig. 16). RT-qPCR analysis showed that CA10g00480 and CA07g02110 genes changed their expression to the greatest extent in response to PSTVd infection (Fig. 16).



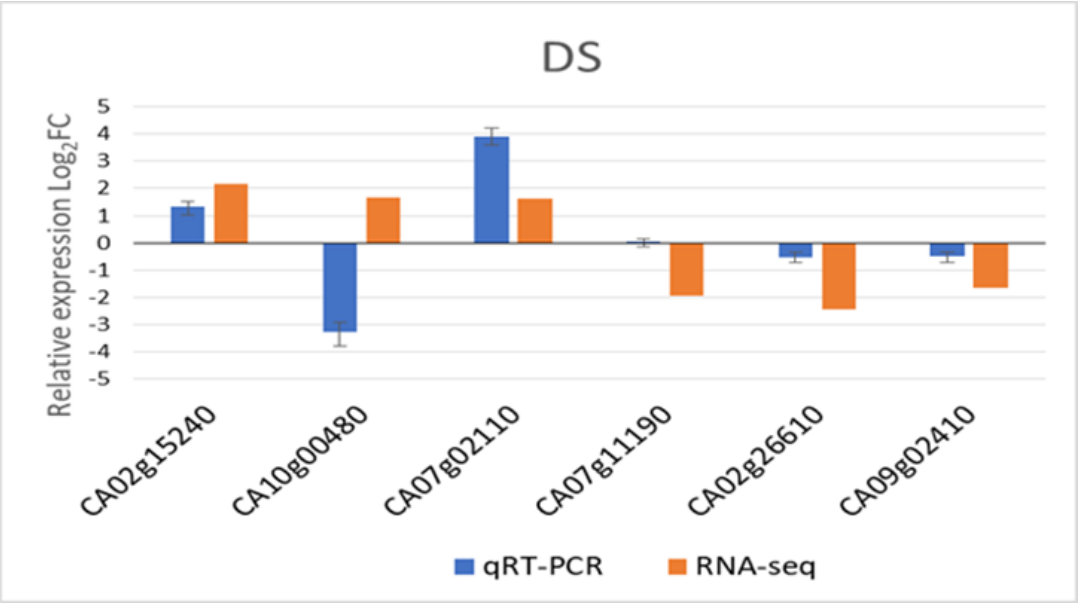


***Fig. 16**: Analysis of the expression profile of selected genes, by RT-qPCR and RNA-seq analysis, in KK and DS, at 43 dpi.*

6.5 Gene Ontology (GO) analysis of overlapping and cultivar-specific DEGs in the two pepper cultivars

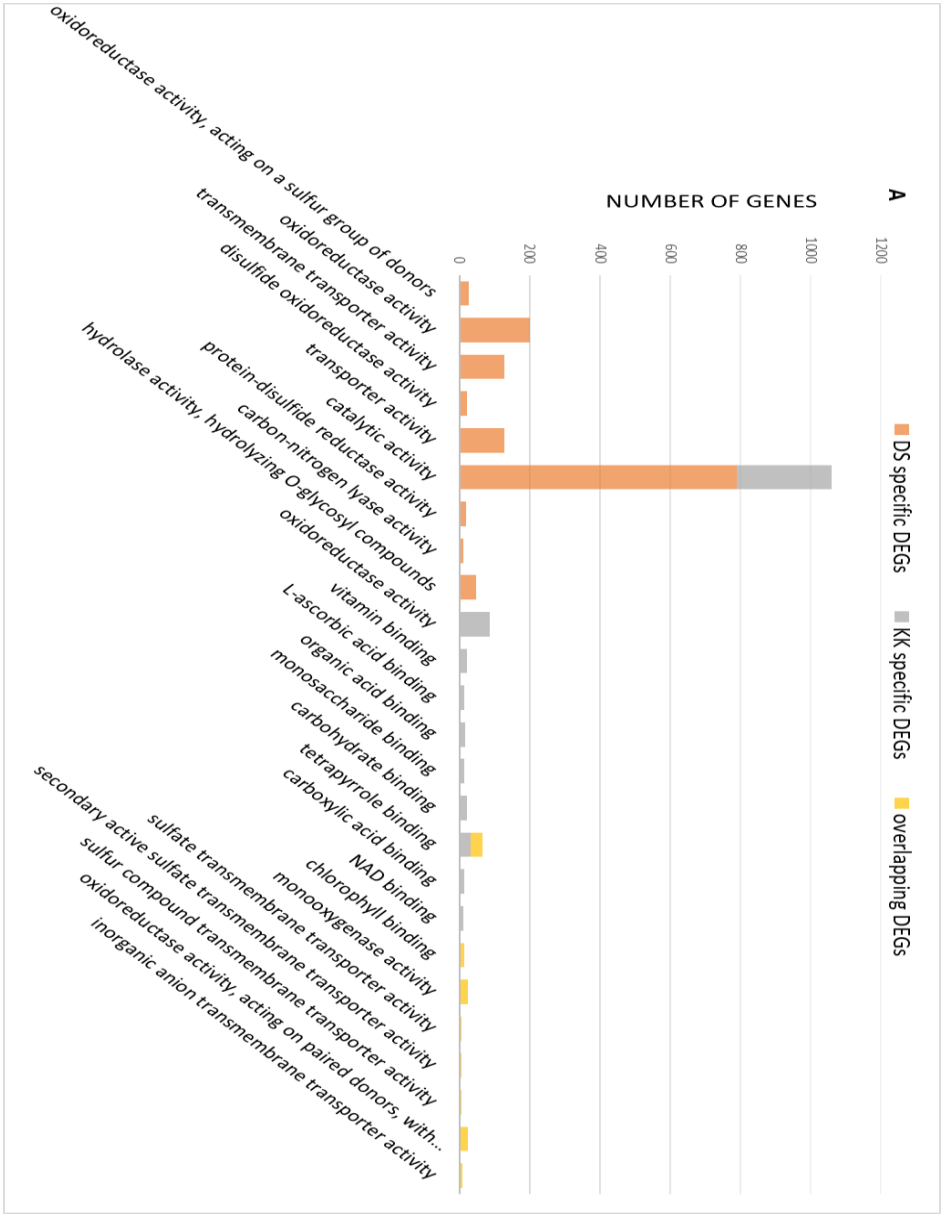
GO analysis of overlapping and cultivar-specific DEGs performed using g:Profiler aims to associate DEGs with specific biological processes (BP), molecular function (MF), or cellular components (CC). Overlapping DEGs were found to include eight MF GO categories, the most abundant of which was chlorophyll-binding, tetrapyrrole-binding, oxidoreductase, and monooxidogenase activity (Fig. 17A). Among the twenty CC GO categories are plastids, chloroplasts, envelopes, organelle envelopes, plastoglobules, photosystems I and II (Fig. 17C), and the fourteen BP GO categories include photosynthesis, light-harvesting complexes of photosystem I, generation of precursor metabolites and energy, response to stimuli and transport (Fig. 17B).

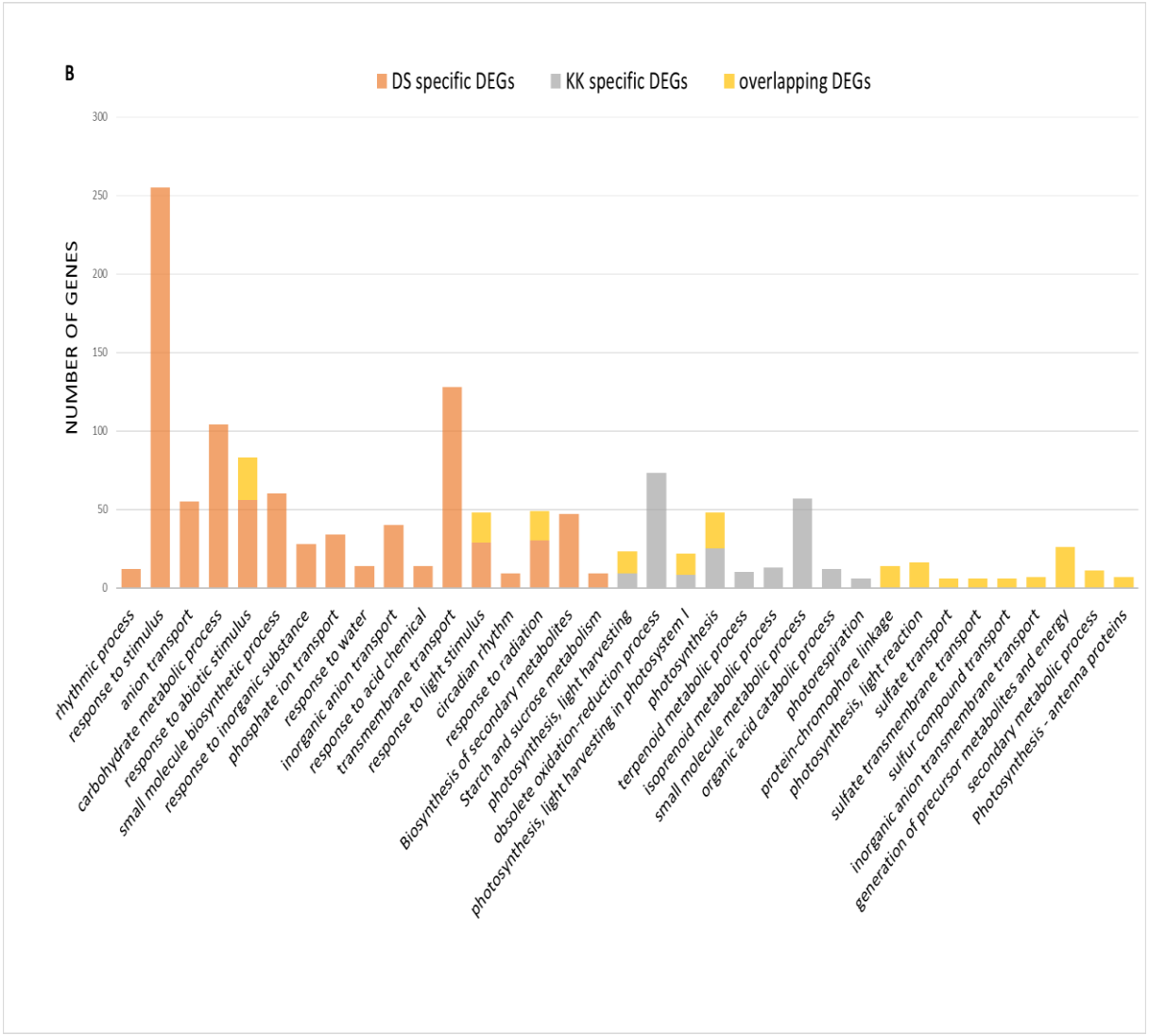
DS-specific DEGs include nine MF GO categories, such as catalytic, oxidoreductase transmembrane transporter activity, and four KK GO categories, among which plastids and chloroplasts are predominant (Fig. 17 A, C); Among the seventeen BP GO categories are responses to stimuli, transmembrane transport, carbohydrate metabolic processes, biosynthetic processes of small molecules, biosynthesis of secondary metabolites, starch and sucrose metabolites (Fig. 17 B).

KK-specific DEGs include 10 MF GO categories, among which are catalytic, oxidoreductase activity, vitamin binding, tetrapyrrole-binding activity (Fig. 17 A); 18 KK GO categories, the most abundant of which are: plastids, chloroplasts, plastid envelope and thylakoid membranes (Fig. 17 C); and 9 BP GO categories including oxidoreductase processes, small molecule metabolic processes, photosynthesis, photorespiration (Fig. 17 B).

In both cultivars, down-regulated genes were found to belong to the BP GO categories-photosynthesis and response to stimuli. Genes with increased expression in response to PSTVd infection in DS were allocated to the category BP GO- defence response, while genes with increased expression

in KK were included in the category BP GO-oxidoreductase processes and response to stimuli (information is presented in supplementary tables attached to the electronic version of the dissertation).




B
DS specific DEGs
KK specific DEGs
overlapping DEGs
NUMBER OF GENES
300
250
200
150
100
50
0
rhythmic process
response to stimulus
anion transport
carbohydrate metabolic process
response to abiotic stimulus
small molecule biosynthetic process
response to inorganic substance
phosphate ion transport
response to water
inorganic anion transport
response to acid chemical
transmembrane transport
response to light stimulus
circadian rhythm
response to radiation
Biosynthesis of secondary metabolites
Starch and sucrose metabolism
photosynthesis, light harvesting
obsolete oxidation-reduction process
photosynthesis, light harvesting in photosystem I
photosynthesis
terpenoid metabolic process
isoprenoid metabolic process
small molecule metabolic process
organic acid catabolic process
photorespiration
protein-chromophore linkage
photosynthesis, light reaction
sulfate transport
sulfate transmembrane transport
sulfur compound transport
inorganic anion transmembrane transport
generation of precursor metabolites and energy
secondary metabolic process
Photosynthesis - antenna proteins

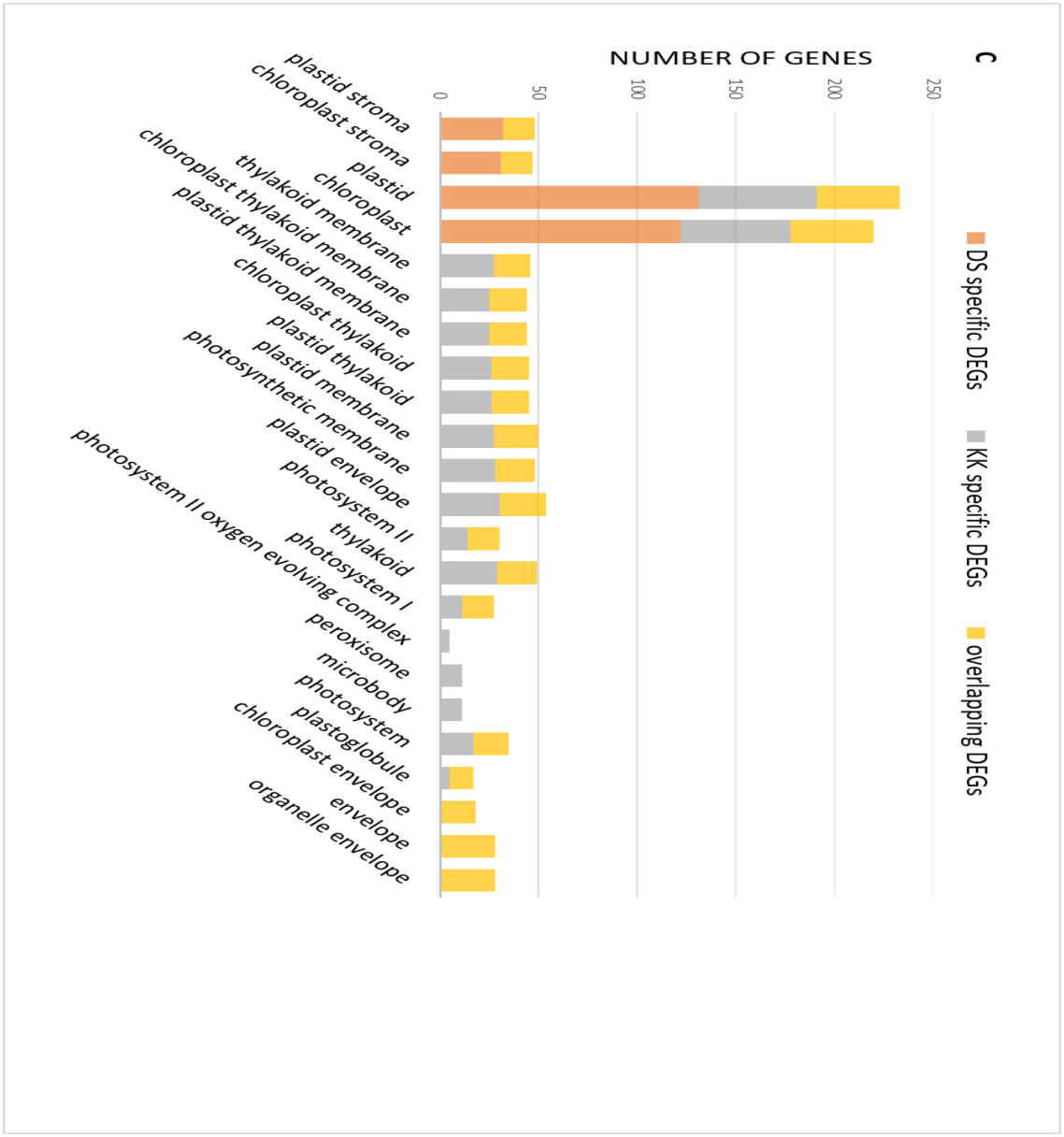


Fig. 17: Gene ontology analysis of DEG in the two pepper cultivars based on g:Profiler (adj p < 0.05). DS-specific, KK-specific and overlapping DEGs are associated with GO categories molecular function (A), biological processes (B), cellular components (C).

The functionally enriched GO categories were also merged using the web-software REVIGO http://revigo.irb.hr/, applying the default parameters (Fig. 16) (Supek et al., 2011).

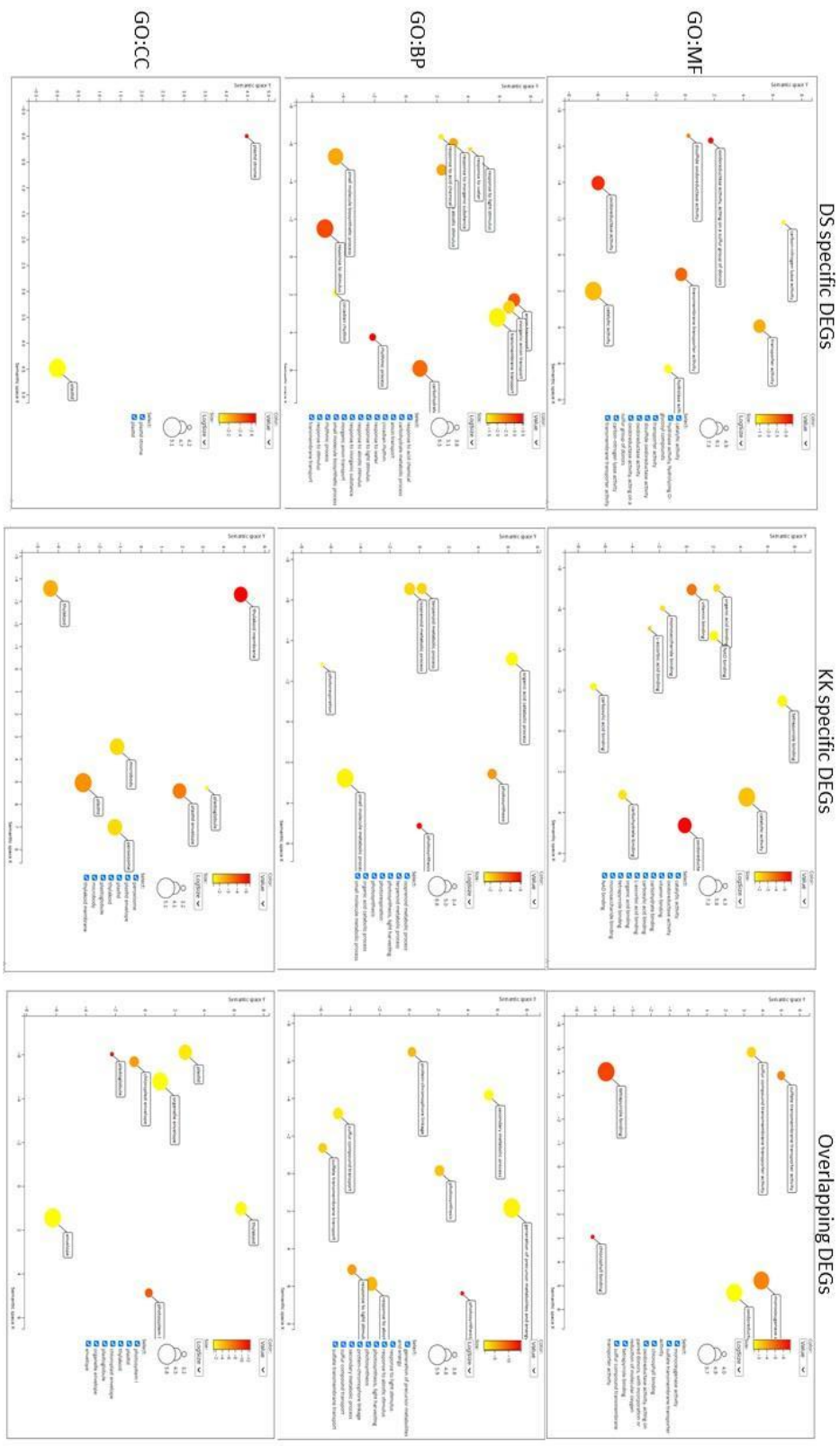


***Fig. 18**: Gene ontology analysis performed using REVIGO (http://revigo.irb.hr/). The x-axis expresses the log10 p-value of each GO category, while the y-axis represents the logarithmic frequency of the GO category in the annotated GO database.*

DISCUSSION

PSTVd infection of different plant species and cultivars of the *Solanaceae* family (tomato, potato, *Solanum laxum*, etc.) has been studied by large-scale gene expression analyses (Li et al., 2016; Kang et al., 2020). However, the molecular basis of pepper (*Capsicum annuum* L.) response to PSTVd infection is still not well explored, which encouraged the use of two Bulgarian pepper cultivars as an experimental system to study PSTVd-pepper relationships. The selection of the two cultivars was based on their agricultural importance and the development of a PSTVd-specific phenotype (Apostolova et al., 2021).

By next-generation sequencing of small RNAs, we were identified >200 miRNAs belonging to >60 families in pepper samples of the two cultivars in response to PSTVd (Fig. 9). Several conserved miRNAs can-miR397, can-miR398, can-miR408, and can-miR482 were found to display cultivar-specific expression in response to PSTVd infection at 28 dpi. In all libraries (mock and infected of both cultivars), 24 nt small RNAs showed the highest abundance, which coincides with the results presented by Hwang and co- authors (2013), who reported the most abundant presence of 24 nt small RNAs in different organs of pepper.

Mapping of small RNAs to the PSTVd KF440-2 genome showed that 21 nt and 22 nt small RNAs predominated in PSTVd infected samples of both cultivars. In PSTVd-infected DS, 21 nt small RNAs were twice as much as in PSTVd-infected KK (Apostolova et al., 2021). Cultivar-specific differences in accumulation patterns of PSTVd-specific 21 nt and 22 nt small RNAs have also been reported in tomato ("Rutger" and "Moneymaker" cultivars) (Wang et al., 2011). The presence of these small interfering RNAs in PSTVd-infected pepper cultivars suggests their active role in plant defense through RNA silencing, similar to that reported in tomato (Papaefthimiou, 2001) as well as in the development of the PSTVd-specific phenotype (Tsushima et al., 2015).

In the present study, several miRNAs can-miR398c-3p, can-miR408a can-miR408-3p, can-miR397b, and can-miR397a-5p, can-miR482a-3p showed opposite expression profiles in the two pepper cultivars in response to PSTVd infection. In plants, miR398 has been shown to be involved in the response to abiotic stress and disease resistance, as it targets genes of the superoxide dismutase (SOD) family: chaperone (CCSD), copper-zinc SOD (CSD) and transcription factors (SOX) (Zhu et al., 2011; Lu et al., 2011). Our expression analyses showed that can-miR398c-3p was induced in PSTVd-infected DS and repressed in KK (Figs 10, 11). This suggests that in pepper, there is cultivar specific expression of miR398 related to defense response to PSTVd (Zhu et al., 2011). Cultivar-specificity regarding the PSTVd response is also found for can-miR482, whose expression is suppressed in DS at 28 dpi, and slightly increased in KK. MiRNAs belonging to the miRNA482/2118 superfamily, miRNA6019, miRNA6024, miRNA6027, regulate genes encoding NB-LRR proteins, which are the major class of genes conferring resistance (R-genes) in tomato, potato, and *N. benthamiana* (Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012; Zhang et al., 2016, Kim, 2019). MiRNA482 modulates resistance in potatoes, by repressing the expression of NBS-LRR genes, upon *Verticillium dahlia infection* (Yang et al., 2015). A similar response was observed in *Fusarium oxysporum* infected tomato (Yang et al., 2021; Ouyang et al., 2014). One of the modes of action by which microRNAs modulate NBS-LRR genes is through specific 22nt miRNAs, which trigger the biogenesis of secondary small interfering RNAs, termed phasiRNAs, and thus increase the silencing of their targets (Ding and Itaya, 2007; Eamens et al., 2014). miR482 has been shown to cause cleavage of the NBS-LRR gene and subsequent production of secondary small RNAs in various plants such as potato, tomato, and tobacco (Yang et al., 2021). Ouyang and co-workers (2014) presented cultivar-specific dynamics of miR482 based on comparative miRNA

analysis in tomato cultivars ‘Moneymaker’ (susceptible to *Fusarium oxysporum*) and ‘Motelle’ (resistant to *F. oxysporum*). They demonstrate impaired resistance to *F. oxysporum* in ‘Moneymaker’ tomato due to the inhibitory effect of miRNAs, sly-miR482f and sly-miR5300, on NB domain-containing proteins. The R3a resistance gene in potato targeted by the miR482 family and results in the production of phasi RNA. Conversely, in tomato R3a homologue I2 is a target of miR6024. Therefore, the evolution of gene families conferring resistance may be due to regulation exerted by different microRNAs (Yang et al., 2021).

Can-miR397a-5p and can-miR408 have increased expression in DS and decreased expression in KK at 28 dpi (Fig. 11). Putative targets of members of the can-miR397 and can-miR408 families are laccase-like genes (Hwang et al., 2013). These genes encode laccases multi-copper oxidases that are involved in lignin and flavonoid biosynthesis and anthocyanin degradation pathways that take part in stress adaptation (Janusz et al., 2020). Our research shows that pepper laccase 4-like and laccase 7-like genes are inversely affected by can-miR397a-5p in both pepper cultivars (Apostolova et al, 2021). From the obtained results and data, collected from other representatives of the *Solanaceae* family during PSTVd infection we can conclude that the phenotypic changes observed in pepper are accompanied by changes in the expression of microRNAs and their target genes (Flores et al., 2012).

Large-scale analyses of PSTVd-infected plants showed significant changes in the expression profiles of protein-coding genes related to hormone biosynthesis (Milanovic et al., 2018) and signaling (Owens et al., 2012), cell wall organization (Itaya et al., 2002) and chloroplast biogenesis and structure. Symptoms such as epinasties and stunting as a result of PSTVd infection are associated with changes in levels of gibberellins, auxins, and brassinosteroids. Proteins of the DRM1/ARPs family are involved in the auxin-mediated signaling pathway. Increasing evidence suggests that DRM1/ARP are involved in the response to abiotic or biotic stress and may be mediators in the response to pathogens and plant growth/development processes (Souza et al., 2019). In most pathogen-related diseases (e.g. disease caused by the fungal pathogen *Fusarium oxysporum* and nematode infection) (De Souza et al., 2019; Kidd et al., 2011), auxin signaling is associated with susceptibility to disease and much less often with disease resistance. The role of auxin in PSTVd-induced defense in plants has been reported by several research groups. Zheng and colleagues reported significant changes in the expression level of 83 genes in tomato related to auxin biogenesis and signaling. Decreased expression of auxin-related genes has been reported in PSTVd-infected plants of the potato cultivar 'Safari' (Katsarou et al., 2016) and the tomato cultivar 'Rutgers' (Wiesyk et al., 2018). Other genes involved in the auxin signaling pathway (eg, ASK2, involved in ubiquitin-mediated proteolysis; IAA3/SHY2, a gene encoding a transcription factor; and IAR1, involved in auxin homeostasis) have been reported to have increased expression in PSTVd-infected tomato cultivar 'Rutgers' (Owens et al., 2012). Altered indole-3-acetic acid (IAA) levels in PSTVd-infected tomato cultivar 'Moneymaker' were associated with loss of apical dominance (Bagherian et al., 2016). Organ-specific changes in the levels of endogenous IAA in PSTVd-infected tubers of potato cultivar 'Désirée' were associated with a change in the expression of genes involved in IAA metabolism (Milanovic et al., 2019).

Transcriptome analysis of PSTVd-infected KK and DS plants showed differential gene expression in response to PSTVd infection, with some of the genes exhibiting opposite expression profiles between the two cultivars. The majority of genes in DS were down-regulated compared to genes in KK, which could explain the more pronounced PSTVd symptoms in DS. The analysis of functional enrichment among the differentially expressed genes shows that most of them with reduced expression in both cultivars are involved in photosynthesis. Genes with increased expression in DS belong to biological processes involved in the defense response. The expression of six differentially expressed

genes in the two cultivars with opposite expression profiles was validated by RT-qPCR. Among the selected genes are those involved in photosynthesis, hormone signaling, and defense pathways. In our analyses, a change in the expression of auxin-related genes was found in PSTVd-infected pepper cultivars. CA02g15240 (auxin-repressed 12.5 kDa protein-like isoform) showed cultivar-specific expression, suggesting the involvement of hormonal control on viroid infection. The increased expression of CA02g15240 in the plants of variety DS can be associated with the higher sensitivity of the variety to the infection and, respectively, the stronger symptomatology in DS.

PSTVd infection has been shown to alter the expression of three genes involved in ethylene biosynthesis, S-adenosylmethionine decarboxylase (SAMDC), aminocyclopropane-1-carboxylic acid synthase (ACS), and (1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO) in tomato (Owens et al., 2012; Wiesyk et al., 2018). SAMDC exhibits dynamics in expression, whereas ACO shows an increase in expression in progress of PSTVd infection (Moeder et al. 2002). Viral infection is known to be associated with increased ethylene synthesis, suggesting that ACO genes are involved in the response against these infectious agents. High accumulation of ST-ACO1 transcripts in potato following PVA (potato virus A) infection (Nie et al. 2002), and NA-ACO1 and NA-ACO3 in TMV infection of tobacco (Kim et al. 1998) were reported. Highly elevated levels of NB-ACO1 were detected in *Nicotiana benthamiana* leaves infected with the fungal pathogen, *Colleotrichum orbiculare* (Shan and Goodwin 2006). Similarly, the accumulation of ACO gene transcripts has been noted in other fungal-plant interactions (Chen et al. 2003; Lahey et al. 2007; Iwai et al. 2006). Studies in NB-ACO1-silenced plants show that the ACO gene is not only induced by pathogen attack but plays an important role in the onset of necrotrophy during fungal infection (Shan and Goodwin 2006; Rudus et al., 2013). Experiments performed on transgenic tomato plants overexpressing VvACO1, and VvACO3 reported normal morphological growth, while the lowest ACO enzyme activities were observed in transgenic tomato lines overexpressing VvACO2, which also showed dwarfing (Tao et al., 2013). In our experiments, the expression of CA07g11190 (ACO) was increased in infected KK plants, suggesting that PSTVd induces ethylene production, leading to an increase in stress tolerance in these plants. In addition, SAMDC is also a key enzyme involved in the biosynthesis of major classes of polyamines, (PAs) that exert pleiotropic effects (Mo et al., 2016) (Ji et al., 2019). Transgenic potatoes in which SAMDC expression is reduced and respectively the PA levels are reduced exhibit a specific phenotype characterized by small leaves and stunted root growth (Kumar et al., 1996). In this regard, a possible reason for the more pronounced phenotype in DS could be the decreased SAMDC expression. Polyamines play a role in ion homeostasis, which could be related to the functional enrichment of DEGs specific for DS in the GO category "Biological processes" - transmembrane transport and anion transport. According to the GO analysis of DEG, several genes with reduced expression involved in the metabolism of phenylpropanoid and cinnamic acid were detected in DS. One of them is an ortholog of PAL, which is involved in phenylpropanoid metabolism and contributes to virus resistance by inducing the accumulation of salicylic acid (SA) (Yuan et al., 2019) (Chen et al., 2006). It has been reported that upon CaPAL1 silencing, pepper plants show reduced SA levels and reduced CaPR1 expression, thus exhibiting greater susceptibility to *Xanthomonas campestris pv. vesicatoria* (Kim et al., 2014). Infection with a highly virulent strain of PSTVd leads to a reduction in PAL expression in tomato plants (Wiesyk et al., 2018). Silencing PAL gene affects plant growth and development. For example, inhibition of PAL expression in tobacco can result in growth retardation, changes in leaf structure, changes in petal morphology and pigment, and reduced pollen viability (Mo et al., 2022). CA09g02410 (PAL) in DS shows reduced expression, which may be the reason for the appearance of the more pronounced PSTVd symptoms.

The impairment in photosynthesis of PSTVd-infected plants is associated with the suppression of genes involved in chloroplast biogenesis and function, such as POR3, Cab4, Cab5, and chloroplast

carbonic anhydrase genes (Itaya et al., 2002). A large number of the genes involved in the photoresponses were down-regulated, as the number of repressed genes in tomato varied depending on the PSTVd strain and the period after inoculation. In confirmation of the reported data, we found a decrease in the expression of the POR gene in both pepper cultivars, according to RT-qPCR. In addition, next-generation mRNA sequencing data showed decreased mRNA levels of other genes involved in chlorophyll biosynthesis (CA04g17210, CA08g13530, CA05g01250, CA10g22340) and Chl a/b binding proteins (CA02g12070, CA07g10990, CA10g02050, CA03g299508, CA04g0608, CA04g09508) in PSTVd infected KK and DS, which together contribute to the development of specific PSTVd symptoms, such as chlorosis at later stages of infection.

Many DS-specific DEGs are associated with the GO category "Biological processes" - response to stimuli. Polygalacturonase inhibiting proteins (PGIPs) respond to multiple stimuli by modifying the expression levels of genes involved in several metabolic pathways, strengthening the cell wall and leading to increased resistance to certain fungal pathogens (Janni et al., 2008; Kalunke et al., 2015; Wang et al., 2013). PGIP genes are involved in basic biological processes such as color development (Gamboa et al., 2001) and response to stimuli (Wang et al., 2013; Ahsan et al., 2005; Cheng et al., 2008; Federici et al., 2006)). Overexpression of PGIP in tomato (*Lycopersicon esculentum*) (Powell et al., 2007) and tobacco (*Nicotiana tabacum*) (Joubert et al., 2006) has been shown to increase resistance to *Botrytis cinerea* infection, while down-regulation increases susceptibility to *B. cinerea* (Ferrari et al., 2007). (Zhang et al., 2016). Heterologous expression of MdPGIP1 gene from *Malus domestica* in tobacco results in the inhibition of PGs from *Colletotrichum lupini*, *Botryosphaeria obtusa* and *Diaporthe ambigua* (Oelofse et al., 2006). In transgenic tobacco, heterologous expression of CaPGIP1 from *Capsicum annuum* resulted in increased resistance to *Alternaria alternata* and *Colletotrichum nicotianae* by reducing their PG activity that causes reduction in the number of infection sites, lesions, and leaf size (Wang et al., 2013). The CA07g02110 (PGIP) expression data which we obtained, showed an opposite expression profile in the two pepper cultivars. However, the role of PGIP in the cultivar-specific response of pepper to PSTVd infection is foreseen.

Multiple genes encoding TFs have shown differential expression in PSTVd-infected tomato and potato cultivars (Zheng et al., 2017; Katsarou et al., 2016; Bao et al., 2019). The data in the present study found a high number of DE TFs in both PSTVd-infected pepper cultivars. The most abundant and with the highest increase in expression DE specific TF in DS are: CA10g14960 (MYB TF), CA04g17920 (C2H2-type zinc finger protein), CA02g04730 (nuclear transcription factor Y subunit A-7-like [NF-YA7 -like]), CA02g01120 (Dof3 protein), and CA09g07450 (ethylene-responsive element binding factor [ERF]). In KK, the DE TFs with greatest change are: CA01g00510 (TT2-like MYB TF), CA09g08120 (WRKY71), CA10g18410 (homeodomain [HD]-related), CA11g12710 (WRKY75-like), CA11g14620 (Myb4- like), CA05g00840 (heat stress transcription factor B-3-like [HsfB3-like]), CA02g05780 (APETALA2 [AP2]/ERF domain-containing TF), and CA03g35110 (DNA-binding protein homolog). Homologous transcripts of MYB genes have been identified in pepper. These transcripts are targets of miRNA families: can-miR159 and can-miR319. The transcription factors GRF, F-box, TCP, and NAM are potential target genes of can-miR396, can-miR394, can-miR319, and can-miR164 (Hwang et al., 2013). Further in-depth analysis of the involvement of miRNAs in the regulation of TFs, mediating the pepper response to PSTVd, should be performed.

Among the overlapping DE TFs, the CA11g16170 gene encoding a BEL-5 transcript showed increased expression. Increased expression of the BEL-5 homologous gene in potato suppresses GC synthesis upon PSTVd infection, thereby affecting tuber formation (Katsarou et al., 2016). This warrants further analysis of the role of CA11g16170 encoding BEL-5 in pepper GC metabolism. In the present study, we find

twelve common DE genes for TF belonging to eight gene families, i.e., C2H2 (CA08g16870), HD-ZIP (CA08g08650, CA02g07270), WRKY (CA09g11950, CA02g01800, CA02g30960), NAC (CA05g04410), NF- YA (CA10g20490), bHLH (CA03g06220, CA04g17180), HSF (CA10g20440), and CO-like (CA02g26690, CA11g01070), which showed opposite expression profiles in the two pepper cultivars. Elucidation of the specific role of each of these TFs in pepper response to PSTVd infection remains to be deciphered in future experiments.

In conclusion, transcriptomic analysis of the two pepper cultivars infected with PSTVd revealed differentially expressed genes that collectively determine the induction of a cultivar-specific response to the pathogen. The cultivar-specific response observed is most likely due to differences in the control of gene expression associated with the limiting of systemic spread of the viroid. The obtained results contribute to a better understanding of the molecular mechanisms controlling the interactions between the viroid and its host and can be applied in the development of strategies to control PSTVd infection among economically important agricultural crops.

CONCLUSIONS

Based on the research conducted in this dissertation, the following conclusions can be drawn:

1. The bioassays performed with PSTVd KF440-2 on DS and KK showed the development of a PSTVd-specific phenotype at the stage 43dpi, with the observed symptoms being more pronounced in DS compared to KK.
2. A higher level of accumulation of PSTVd (+) RNA was found in DS, which could explain the more pronounced PSTVd symptoms in this cultivar.
3. Distribution of PSTVd specific RNAs in the analyzed libraries of of DS and KK showed twice as many 21nt sRNA in DSI compared to KKI.
4. By means of next-generation sequencing of small RNAs, 14 differentially expressed miRNA were identified in KK and 32 in DS, respectively, in response to PSTVd infection.
5. Expression analysis by RT-qPCR of selected common DE miRNA with opposite expression profiles suggested a cultivar-specific response to PSTVd infection.
6. Next-generation sequencing of mRNA in the analyzed pepper libraries showed three times more specific DEGs in DS compared to KK.
7. Of the overlapping DEGs, 224 genes exhibited opposite expression profiles in the two cultivars and were suggested to collectively determine the induction of a cultivar-specific response to PSTVd.
8. Twenty-three transcription factors were identified among the overlapping DEGs. Twelve of the overlapping DE TF genes showed opposite expression profiles in the two pepper cultivars.
9. The GO analysis of the overlapping DEGs groups the genes in: 14 GO categories of biological processes, the main of which is photosynthesis; 8 GO categories - molecular functions, the most abundant of which is chlorophyll-binding activity and 20 GO categories - cellular components, among which plastids are predominant.
10. The GO analysis of the specific DEGs in the two cultivars shows that the genes with reduced expression belong to the GO categories BP - photosynthesis and response to stimuli; the genes with increased expression in response to PSTVd infection in DS are included to the GO category BP - defense response, while those with increased expression in KK are included in the GO category BP-oxidoreductase processes and response to stimuli.

SCIENTIFIC CONTRIBUTIONS

1. For the first time, the molecular interactions between PSTVd and two cultivars of Bulgarian pepper were investigated by large-scale expression analyzes of smallRNAs and mRNAs.
2. For the first time, differentially expressed miRNAs and differentially expressed protein-coding genes were identified in pepper cultivars KK and DS, which collectively determine the induction of a cultivar-specific response to PSTVd.
3. The described molecular interactions between PSTVd and pepper, suggest their role in modulating viroid spread, replication, and development of viroid-specific symptoms, which may contribute to the development of strategies to improve the control of PSTVd infection in agricultural crops.
4. Results of large-scale sequencing of mRNA obtained from infected and mock pepper plants are annotated in the NCBI biodatabase with accession number: PRJNA762255 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA762255.
5. The results of the large-scale sequencing of small RNAs of this dissertation are annotated in the NCBI SRA biodatabase: https://dataview.ncbi.nlm.nih.gov/object/PRJNA631129?reviewer=l0686rrde7fsor65p7j623vadd

DISSERTATION RELATED PUBLICATIONS

1. Hadjieva N., Apostolova E., Baev V., Yahubyan G., Gozmanova M. Transcriptome Analysis Reveals Dynamic Cultivar-dependent Patterns of Gene Expression in Potato Spindle Tuber Viroid-infected Pepper, Plants, MDPI, Special Issue "High-Throughput Sequencing Applied to Plant Virus and Viroid Detection" Plants 2021, 10, 2687. https://doi.org/10.3390/plants10122687 . Q1, IF (2021) 3.935

2. Apostolova E., N Hadjieva, D. Ivanova, G. Yahubyan, V. Baev, M. Gozmanova. MicroRNA expression dynamics reshape the cultivar-specific response of pepper (*Capsicum annuum* L.) to potato spindle tuber viroid (PSTVd) infection, Scientia Horticulturae, Volume 278, 2021, 109845, ISSN 0304-4238, https://doi.org/ 10.1016/j.scienta.2020.109845. Q1, IF (2021) 3.463

3. Apostolova E., Hadjieva N., Ivanova D., Tomlekova N., Yahubyan G., Baev V., Gozmanova M. Characterization of small rnas in the pstvd-induced response of two bulgarian pepper cultivars, Scientific works of the Union of Scientists in Bulgaria Plovdiv. Series C. Technique and technologies. Volume XVIII, ISSN 1311 -9419 (Print); ISSN 2534-9384 (Online), 2020. Scientific Works of the Union of Scientists in Bulgaria - Plovdiv. Series C. Technics and Technologies. Vol. XVIII, ISSN 1311 -9419 (Print); ISSN 2534-9384 (Online), 2020. https://usb-plovdiv.org/scientific-works/

LITERATURE

A complete list of references can be read in the dissertation.