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**The Thesis of the PhD dissertation**

# Sweet Potato Viruses: Detection, Elimination and Transcriptome Analysis of Resistance Mechanism in Co-Infected Plants

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## BACKGROUND

Sweet potato (*Ipomoea batatas* (L.) Lam, family Convolvulaceae) is the third most important root and tuber crop globally and one of the most important staples in Sub-Saharan Africa. It is a highly fibrous and nutritious crop with pharmaceutical and ornamental values. The anthocyanin rich purple fleshed cultivars are rich in cancer preventing antioxidants, while the orange fleshed are rich in vitamin A pre-cursor beta-carotene, which is valuable for the prevention of blindness. The high fibre content and low glycaemic index of sweet potatoes are good for prevention of obesity and diabetes (Loebenstein and Thottappilly, 2009; Khoo et al., 2017).

Between 2010-2020, the global sweet potato production reduced by 4.9% to 89 million tonnes, while the harvested area reduced by 7.2% to 7.4 million hectares (FAO, 2022).

Globally, over 30 viruses affect sweet potatoes (Untiveros et al., 2007; Clark et al., 2012; Liu et al., 2020). It is critical that the propagation materials are free of viruses to avoid losses in yields and farmers' profits.

Sweet potato is a hexaploid and heterozygous plant. It is incompatible to self and cross pollination, making conventional breeding difficult. Therefore, developing resistant cultivars is the most effective control method for viral diseases (Okada et al., 2001; Loebenstein, 2012; Sivparsad and Gubba, 2014; Bhat et al., 2016). CRISPR-Cas13 constructs developed to target SPSCV-RNase3 enhanced resistance of sweet potatoes to SPVD (Yu et al., 2022).

Low et al., (2017) recommend propagation of sweet potato cultivars that will benefit farmers most as they deal with the effects of climate change. These ought to be nutritious and tolerant to drought, pests, and diseases, such as Tio Joe and Melinda cultivars (Musembi et al., 2019).

## AIMS

1. **To detect viruses infecting sweet potatoes in Hungary.**

   To find viruses infecting sweet potato germplasm in Hungary, sweet potato samples will be collected from different parts of the country and checked for fifteen important viruses using molecular tests (PCR, qPCR) and bioassay.

2. **To eliminate viruses from local sweet potato cultivars in Hungary.**

   Heat treatment and meristem tip culture will be employed to remove viruses from selected farmers-preferred sweet potato cultivars in Hungary.

3. **Transcriptome analysis to elucidate the mechanism of resistance and susceptibility to sweet potato pakakuy virus (SPPV) and sweet potato chlorotic stunt virus (SPCSV) co-infection.**

   Pathogen-tested sweet potatoes will be graft-inoculated with SPPV-SPCSV to find resistant and susceptible cultivars to SPPV-SPCSV co-infection. Transcriptome of the resistant and susceptible plants will be analysed to identify putative resistance genes and the resistance mechanism.

## MATERIALS AND METHODS

### i. Sources of plant materials

In Hungary, we collected 62 symptomatic and 38 symptomless sweet potato vines from farmers' fields in Galgahévíz, Ásotthalom and Szeged; and storage roots from researchers in Szeged and Gödöllő; national gene bank in Tápiószele; and retail stores in Budakeszi and Berzence between 2019 and 2021.

Eighteen PT sweet potato cultivars were obtained in January 2020 from the International Potato Center (CIP) in Nairobi.

One plant collected from Ásotthalom, Hungary and labelled *A6.1* had severe disease symptoms in the greenhouse. It was later found to be infected with SPSCV and SPPV only. Due to the economic and phytosanitary importance of these two viruses, *A6.1* was propagated in the greenhouse and *in vitro* as a virus inoculum source.

### ii. DNA and RNA extraction

For virus detection, leaf discs were cut from near the petiole of each plant sample's top, middle and lower leaf. For resistance screening, leaf samples were collected from each scion's top fully open leaf, and total RNA isolated using SV total RNA isolation kit (Promega, Madison, USA).

Trizolate reagent (UD-GenoMed, Debrecen, Hungary) was used to isolate RNA.

The quality and quantity of the DNA and RNA were checked using a NanoDrop® spectrophotometer ND-1000 (Thermo Scientific, Wilmington, USA) and 1% agarose gel.

### iii. Primer design

Genomic and coding sequences for fifteen viruses and different genes of interest were obtained from the GenBank and aligned in SeqMan Pro (v. 7.1.0, 44.1) to design primers. Primers were designed in Primer3web version 4.1.0 and Lasergene PrimerSelect (v. 7.1.0, 44) to amplify the viruses and the genes.

### iv. PCR and qPCR

The nucleic acids extracted were diluted to optimal concentrations (10–50 ng/µl) for PCR and qPCR. DNA viruses were tested by PCR using DreamTaq DNA Polymerase

(Thermo Scientific, Vilnius, Lithuania) and universal primers for the respective viruses. RNA viruses were tested by qPCR using qPCRBIO SyGreen one-step qPCR kit (PCR Biosystems, London, UK) and both specific and universal primers for the respective viruses. Both PCR and qPCR were followed by 1% agarose gel electrophoresis.

cDNA of each RNA virus detected was prepared (from randomly selected positive samples) using RevertAid first strand cDNA synthesis kit (Thermo Scientific, Vilnius, Lithuania), then amplified by PCR.

Sweet potato chlorotic stunt virus RNA1 and RNA2 complete genome sequences from sample *A6.1* were obtained via PCR amplifications of cDNA using primers.

For virus quantification and gene expression analysis, cDNA was prepared from the total RNA using the RevertAid first strand cDNA synthesis kit and random hexamers following manufacturer's instructions.

Amplification of viruses and genes of interest by qPCR was performed with two technical replicates. Sweet potato actin was used for normalisation to neutralise differences in sample quantities. The amplified virus fragments were purified and sequenced either directly or after cloning, and the sequences searched in the GenBank to confirm the virus identity.

**v. Virus elimination**

Vines were obtained from each of the four cultivars labelled *T96, 92R, 105R* and *12R* from the National Centre for Biodiversity and Gene Conservation of Hungary and two labelled *Blk* and *Ylw* provided by a producer farmer. Thirty, two-weeks old progenies from each of the six cultivars were heat treated in a versatile environmental test chamber (Growth chamber, model MLR-350, Sanyo, Japan) at $25^0$C for 7 days, $29^0$C for 14 days and $39^0$C for 28 days to free them of viruses (Dennien et al., 2013). Surviving shoot tips (2 cm) were cut out, washed and sterilised. After that, meristem tips were carefully cut out and cultured in half-strength Murashige and Skoog (MS) media, where they formed calluses, which developed shoots and roots within 8-12 weeks. The plantlets were multiplied *in vitro,* and progenies acclimatised in soil and

then transferred to the greenhouse. After eight weeks, they were tested for viruses by PCR or qPCR. Those that were negative for all viruses except SPPV, which was persistent in all the plants, were grafted to *I. setosa* to confirm the absence of the viruses.

**vi. Bioassay**

Wedge and side grafts were made to enhance virus transmission to the indicator plant. An *I. setosa* grafted with a virus-free sweet potato scion served as a negative control. Symptoms were evaluated in *I. setosa* plants for eight weeks and recorded. DNA and RNA were extracted from leaves of the grafted *I. setosa* to test for viruses by PCR and qPCR.

**vii. Resistance screening to SPPV-SPCSV co-infection**

The SPPV-SPCSV infected *A6.1* was propagated for two months alongside the PT cultivars before wedge grafting of three biological replicates with one control at 6-8 nodes length. Leaf samples were collected for DNA and RNA extraction in the 1$^{st}$ and 3$^{rd}$ wpi.

**viii. RNAi genes expression analysis**

RNAi genes expression analysis was carried out in Melinda and Tio Joe cultivars for DCL2, DCL4, AGO1, AGO4 and SDE5 by qPCR using the delt-delta Cq method to investigate gene silencing during SPPV-SPCSV co-infection.

**ix. Transcriptome analysis**

Two treated biological replicates of the resistant Tio Joe and susceptible Melinda cultivars, which showed similar phenotypic (symptoms) and genotypic (gene expression, virus accumulation) characteristics, were selected and pooled for high throughput sequencing. Four libraries of treatments and four mocks labelled ME_1, ME_3, TJ_1 and TJ_3, where ME – Melinda, TJ – Tio Joe, 1,3 – wpi were prepared. Illumina sequencing was done after rRNA depletion in the total RNA (IbioScience, Pécs, Hungary).

Trimmed reads were mapped using default settings in CLC Genomics Workbench v 21.0.5 (QIAGEN, Aarhus, Denmark) to *Ipomoea trifida* genome (NSP306, Hard

Masked Genome Assembly, v3) (Wu et al., 2018). Unmapped reads were *de novo* assembled and then searched for viruses using a database of all viruses downloaded on 23 December 2022 from GenBank.

Differential gene expression (DGE) analysis compared treatments to their respective mocks. Gene set enrichment analysis was performed for differentially expressed genes (DEGs) with mean expression values above 5.0,

Six randomly selected DEGs were validated by qPCR in the three biological replicates of ME and TJ treatments and their mocks with two technical replicates. Sweet potato actin was used as a reference gene for normalisation, and relative gene expression was calculated using the delta-delta Cq method.

## RESULTS AND DISCUSSION

### i. Virus detection

We tested 110 plants from seven sweet potato growing regions in Hungary for 15 virus species (4 DNA viruses, 11 RNA viruses) belonging to nine. Seven viruses were detected: SPCSV, SPFMV, SPV2, SPVC, SPVG, SPLCV and SPPV. This is the first report on the occurrence of SPCSV (F. W. Kiemo et al., 2022), SPPV, SPLCV and sweet potato virus disease (SPVD) caused by SPFMV and SPCSV synergistic infection in sweet potatoes in Hungary (Francis W. Kiemo et al., 2022).

Potyviruses caused chlorotic spots, while SPFMV caused feathery mottles in the veins. SPPV and SPLCV were mostly symptomless. Infection of SPCSV with other viruses caused the most severe symptoms due to synergism. There was no correlation between symptom severity and the number of viruses in a plant. The severity of the symptoms could be associated with the virus combination and their titres (Gibson et al., 1998; Mukasa et al., 2006; Untiveros et al., 2007; Liu et al., 2020). Symptomless infections cause virus prevalence as farmers and producers unknowingly select infected plants for propagation (Gibson et al., 1997; Gibson and Kreuze, 2015; Kreuze et al., 2020). Virus accumulation results in cultivar degeneration which was depicted by the low number of plants (n=2) infected with six viruses (Gibson and Kreuze, 2015).

*I. setosa* is sensitive to known sweet potato viruses. The high concentrations of viruses accumulate in the indicator plant and cause apparent symptoms. Virus diagnosis was easier in *I. setosa* because of the high virus titres and lack of PCR inhibiting latex and phenolic compounds like in *I. batatas* (Kokkinos and Clark, 2006b; Valverde et al., 2007). PCR and qPCR are highly sensitive and can detect viruses in very low titres, such as SPPV, which can be less than one copy in a cell (Kokkinos and Clark, 2006b; Kreuze et al., 2020).

### ii. Virus elimination

There is no system to provide farmers with or ensure that sweet potato propagation materials are PT in Hungary. Farmers in Hungary store harvested roots as propagation material for the next season (Monostori and Szarvas, 2015), leading to persistence and accumulation of viruses in the crop (Gibson et al., 1997). Planting PT sweet potatoes will increase yield and prevent cultivar degeneration due to virus accumulation (Beetham and Mason, 1992; Gibson and Kreuze, 2015).

We successfully eliminated five viruses (SPFMV, SPV2, SPVC, SPVG and SPLCV) from five local sweet potato cultivars (*T96, 92R, 12R, Blk* and *Ylw*); Therefore, providing an impetus for setting up a national or regional system for producing PT sweet potato propagation materials in Hungary. Rukarwa et al., (2011) attained over 70% plant recovery and virus elimination after four weeks of heat treatment (36ºC/16 hours and 32ºC/8 hours daily) and meristem tip culture of *in vitro* sweet potatoes. This rate is much higher than the 50% recovery from heat treatment and 13% virus elimination we achieved. Perhaps virus elimination is easier from *in vitro* plants than potted plants (Rukarwa et al., 2011; Wang et al., 2018). Successful virus elimination in sweet potatoes depends on the cultivar, viruses present, treatment plan and precision in cutting meristem tips (Rukarwa et al., 2011; Dennien et al., 2013; Wang et al., 2018). An extended high temperature eliminates viruses best but reduces plant survival (Kidulile et al., 2018; Wang et al., 2018).

SPPV persisted after heat treatment and meristem tip culture, consistent with observations of Kreuze et al., (2020). Heat treatment can eliminate viruses in the phloem, such as SPLCV, with less difficulty than those in the meristems like SPPV. Perhaps SPPV would be removed by thermotherapy coupled with chemotherapy or cryotherapy (Wang et al., 2018).

The symptomless infection, low titre and tenacity in the cytoplasm of meristematic cells after heat treatment suggest that SPPV is a persistent virus causing latent infection in sweet potatoes (Roossinck, 2012; Takahashi et al., 2019; Kreuze et al., 2020; Bradamante et al., 2021; Francis W. Kiemo et al., 2022). SPPV-sweet potato

relationship is possibly symbiotic since the virus is 'allowed' to invade the seeds and meristematic cells of sweet potatoes (Roossinck, 2008, 2012; Takahashi et al., 2019; Kreuze et al., 2020). RNAi makes the meristem invasion and recovery from SPPV possible (Bradamante et al., 2021).

The inability to directly detect all viruses in sweet potatoes upholds the importance of biological assay (Kokkinos and Clark, 2006a). Nonetheless, the absence of SPPV in most *I. setosa* grafted with scions containing the virus raises serious concerns as it affects the integrity of PT or 'virus-free' plants (Francis W. Kiemo et al., 2022).

**iii. Resistance screening to SPPV-SPCSV co-infection**

Five weeks after inoculation, symptoms gradually developed in ME, Mugande and *Ylw* from the fifth wpi correlating with the high virus titres recorded at six and nine wpi. These cultivars were therefore deemed susceptible to the SPPV-SPCSV co-infection (Tavantzis, 1984; Untiveros et al., 2007). A confirmatory test also recorded higher virus titres in ME than in TJ. TJ was the only cultivar lacking virus symptoms and gradually decreased SPPV and SPCSV concentrations from the third to the twelfth wpi; hence, it was considered resistant to the dual virus infection (Tavantzis, 1984; Loebenstein and Carr, 2006).

A positive correlation coefficient of 0.7 between SPCSV and SPPV titres suggests synergism. Kreuze et al., (2020) reported increased SPPV siRNA in plants co-infected with SPCSV.

**iv. DEGs responsive to viruses**

The presence of SPPV in the mocks negates its role in differential gene expression between the mocks and treatments. Consequently, the differential gene expression could be attributed to SPCSV introduction.

RNAi genes DCL4 and SGS3 were upregulated in TJ_1. DCL4 cleaves viral mRNA and dsRNA to vsiRNA, while SGS3 amplifies the tasiRNAs during gene silencing (Csorba et al., 2015). Low expression of DCL2, DCL4, AGO1, AGO4 and SDE5 could be to balance growth and defence responses after SPPV infection. Surprisingly, SPCSV introduction did not elevate gene silencing. We suspect that SPPV induced

RNAi before grafting to prime the plants' defence against viruses through cross-protection (Ryals et al., 1994; Loebenstein and Carr, 2006). Systemic priming of AGO2 was induced through systemic acquired resistance (SAR) by cucumber mosaic virus in resistant Arabidopsis (Ando et al., 2021).

Many genes commonly induced or targeted by viruses were differentially expressed (Whitham et al., 2006). In ME_1, pathogenesis-related family protein was the most highly upregulated gene in response to virus infection, while another gene, namely pathogenesis-related thaumatin superfamily protein, was the most downregulated DEG. Overexpression of pathogenesis-related genes is associated with compatible plant-virus interactions that lead to systemic spread of the viruses (Maule et al., 2002).

The white-brown complex homolog protein (ABCG11) was upregulated in ME_1 and ME_3. It is an ABC-2 type transporter of wax and cutin, which makes cuticle that reduces transpiration and prevents pathogen entry (Bird, 2008). Putative mitochondrial RNA helicase was upregulated in both cultivars, one wpi. Jasmonic acid (JA) and salicylic acid (SA) trigger RNA helicase production in response to biotic and oxidative stresses to promote RNA metabolism, transcription and translation. Viruses hijack RNA helicase for their replication or to suppress RNA silencing; nonetheless, upon interacting with viral dsRNA, RNA helicase triggers an antiviral signal that leads to gene silencing of the virus (Ranji and Boris-Lawrie, 2010).

### v. Putative SPPV-SPCSV resistance genes

The DEGs overexpressed in TJ more than in ME could be responsible for low SPCSV titre in TJ and hence qualify as putative disease resistance genes to SPPV-SPCSV co-infection. The resistance mechanism against SPPV-SPCSV co-infection in TJ could have involved:

- Nodulin MtN21/EamA-like transporter family protein transporting amino acids (such as glutamine and histidine) and auxins in the vascular tissue to form secondary cell wall (Vanholme et al., 2010).

- SAUR-like auxin-responsive protein inducing cell elongation and growth through acidifying cell walls. It prevents dephosphorylation of plasma membrane $H^+$-ATPase, which induces expression of SA and pathogenesis-related genes early in the infection (Schaller and Oecking, 1999; Elmore and Coaker, 2011).
- Metallothionein 2A scavenging of ROS to reduce oxidative stress that could damage the infected cells (Patankar et al., 2019).
- Strengthening the cell wall through lignification by cinnamoyl-CoA reductase (CCR)-like gene (Bart et al., 2010)

**vi. KEGG pathways**

Enriched KEGG pathways include vitamins and phenolic compounds, which are antioxidants that modulate ROS in infected cells. Resistance to tobacco mosaic virus is increased by overexpression of antioxidants (Dutilleul et al., 2003). Vitamin B6 is a cofactor in amino acid biosynthesis reactions. Thiamine induces resistance to pepper mild mottle virus in tobacco through SA and calcium ions ($Ca^{2+}$) signalling (Ahn et al., 2005; Denslow et al., 2005; Boubakri et al., 2016). Nicotinate and nicotinamide (vitamin B3) are used in nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+) biosynthesis. NAD+ and NADP+ are coenzymes in homeostatic reactions used to regulate ROS accumulation, repair DNA, increase amino acids and ATP production, and induce SA defence responses (Hashida et al., 2010; Pétriacq et al., 2013).

Products of enriched sucrose metabolism and phenylpropanoid biosynthesis are used in biosynthesis of secondary metabolites such as hexoses and flavonoids essential for defence. Apoplast sucrose levels regulate cell wall invertase production of hexoses which induce signal transduction during defence (Proels and Hückelhoven, 2014; Singh and Singh, 2018).

Enriched mitogen activated protein kinase (MAPKs) are activated by and respond to diverse biotic and abiotic stimuli through crosstalk with phytohormones. MAPKs as transcription factors can activate defence genes and induce biosynthesis of phenolic

compounds (Bigeard and Hirt, 2018; Jagodzik et al., 2018; Singh and Singh, 2018). MAPK phosphorylation of a disease resistance gene in tobacco activates resistance to tobacco mosaic virus (Bigeard and Hirt, 2018).

### vii. SPPV-SPCSV resistance mechanism

Virus inhibition and lack of symptoms in TJ were consistent with resistance (Tavantzis, 1984; Karyeija et al., 1998). We hypothesise that the resistance mechanism against SPPV-SPCSV co-infection involves SAR and recessive resistance. SAR employs JA, SA and ET to inhibit virus spread and prime parts of the plant far from the infection site for defence against the invading pathogen through the production of pathogenesis-related (PR) genes, cell wall strengthening and biosynthesis of secondary metabolites (Ryals et al., 1994; Soosaar et al., 2005; Carr et al., 2010; Zvereva and Pooggin, 2012). Recessive resistance commonly associated with eIF4E, which was overexpressed in TJ, is more durable and capable of inhibiting virus accumulation for as long as the virus does not adapt to multiplication or movement without the missing cofactor (Carr et al., 2010; Hashimoto et al., 2016). Functional genomics analysis will help to understand the resistance mechanism in TJ and verify the suggested putative resistance genes.

## CONCLUSION AND RECOMMENDATIONS

Sweet potatoes were collected from various sources in Hungary and evaluated for fifteen important viruses. Five RNA viruses were detected by qPCR: SPCSV, SPVG, SPVC, SPFMV, and SPV2. PCR detected two DNA viruses: SPLCV and SPPV. We have reported for the first time in Hungary the occurrence of three viruses, SPCSV, SPPV and SPLCV and the worst disease of sweet potato, sweet potato virus disease (SPVD), caused by co-infection of SPFMV and SPCSV. The lack of virus-free sweet potato propagation materials was a key contributor to the spread of these viruses in farmers' fields. We successfully eliminated five viruses (SPFMV, SPV2, SPVC, SPVG and SPLCV) from five local sweet potato cultivars (T96, 92R, 12R, Blk and Ylw); Therefore, providing an impetus for setting up a national or reginal system for producing PT sweet potato propagation materials in Hungary.

SPPV is a persistent virus that is hardly removed by heat treatment or meristem tip culture and cannot be easily transmitted by grafting to the *I. setosa* indicator plant. It could only be detected in 14% of *I. setosa* grafted with scions containing the virus.

It's almost universal presence in our samples, and global sweet potato germplasm is a phytosanitary challenge, especially for the international transfer of germplasm.

A severely diseased plant infected with SPPV and SPCSV was collected from a farmer's field in the south of Hungary. Alone, SPPV and SPCSV do not cause much damage to sweet potatoes. Therefore, we decided to investigate the mechanism of resistance or susceptibility to SPPV-SPCSV co-infection in sweet potatoes. Cultivar Melinda, Mugande and *Ylw* developed virus symptoms in the growth chamber from five wpi after grafting to SPPV-SPCSV infected rootstock. They had high virus titres and hence were deemed susceptible. Tio Joe cultivar was symptomless, and its virus titres gradually reduced up to twelve wpi in the first test. At three wpi in the second test, the average SPCSV titre was eight-fold higher in ME than in TJ. Tio Joe was therefore considered resistant to SPPV-SPCSV co-infection based on its ability to inhibit virus accumulation and lack of symptoms. A positive correlation coefficient

of 0.7 between SPPV and SPSCV suggests synergism, although SPPV titres were always much lower than SPCSV.

Pre-infection of SPPV might have influenced our transcriptome analysis by inducing defence responses in both mock and treatments, making the introduction of SPCSV responsible for the symptoms and differential gene expression. DEGs that responded to virus infection were mostly SAR genes and virus cofactors. They include white-brown complex homolog protein, putative mitochondrial RNA helicase and pathogenesis-related proteins, which were validated. The DEGs overexpressed in TJ more than in ME significantly reduced the replication and spread of SPCSV in TJ and could be responsible for the SPPV-SPCSV resistance. They include nodulin MtN21 /EamA-like transporter family protein, SAUR-like auxin-responsive protein family, Metallothionein 2A and CCR-like, validated by qPCR. Functional genomics analysis of these genes will give a comprehensive view of how the viruses interacted with each other and their hosts during the co-infection, which could help in molecular breeding for resistance. Based on the transcriptome analysis, SAR and recessive resistance are the probable mechanisms for SPPV-SPCSV resistance in TJ.

Overall, this study highlights the importance of planting virus-free sweet potatoes and understanding the resistance mechanisms in complex virus infections to prevent and control viral diseases in sweet potatoes. It is recommended that the spread and economic significance of these viruses in Europe be investigated; a PT sweet potato production scheme be set up in sweet potato growing regions of Europe; farmers be educated to avoid planting sweet potatoes meant for food from the retail stores; and finally, plant health authorities to strictly regulate international germplasm movement.

## NEW SCIENTIFIC RESULTS

1. First report of the occurrence of sweet potato virus disease (SPVD), sweet potato chlorotic stunt virus (SPCSV), sweet potato leaf curl virus (SPLCV) and sweet potato pakakuy virus (SPPV) infecting sweet potatoes in Hungary.
2. Eliminated SPFMV, SPVG, SPVC, SPV2 and SPLCV from two sweet potato cultivars (labelled: *Blk* and *Ylw*) from farmers and three (labelled: T96, 92R, 12R) from the National Centre for Biodiversity and Gene Conservation of Hungary.
3. First report of graft transmission of SPPV from sweet potato to *I. setosa*.
4. Molecular characterisation of SPPV-SPCSV co-infection in sweet potato cultivars showing severe symptoms in the field and greenhouse.
5. Comprehensive transcriptome analysis and discussion of resistance to SPPV-SPCSV co-infection.

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