Hungarian University of Agriculture and Life Sciences

Doctoral (PhD) Dissertation

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

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ABA: Abscisic acid

ABC: ATP-binding cassette

ACC: Accession

ACT: **A**spartate kinase, **C**horismate mutase, **T**yrA (prephenate dehydrogenase)

AGO: Argonaute

ATP: Adenosine triphosphate

BURP: **B**NM2 - *Brassica napus* microsporogenesis-specific protein; **U**SP - *Vicia faba* unknown seed protein; **R**D22 - *Arabidopsis thaliana* gene Responsive to Dehydration 22 and **P**olygalacturonase isozyme

CCR: Cinnamoyl-CoA reductase

CIP: International Potato Center

DAVID: Database for Annotation, Visualization and Integrated Discovery

DCL: Dicer-like enzymes

DEGs: Differentially expressed genes

dsDNA: Double stranded DNA

dsRNA: Double stranded RNA

eIF4E: Eukaryotic Initiation factor 4E

ET: Ethylene

FDR: False discovery rate

Hsp70: Heat shock protein 70

IAA: Indole-3-acetic acid

JA: Jasmonic acid

KEGG: Kyoto Encyclopedia of Genes and Genomes

MAPK: Mitogen activated protein kinase

ME: Melinda sweet potato cultivar

mRNA: Messenger RNA

MtN21: *Medicago truncatula* nodulin 21

NAD: Nicotinamide adenine dinucleotide

PCR: Polymerase chain reaction

PR: Pathogenesis-related

PT: Pathogen-tested

PTGS: Post-transcriptional gene silencing

qPCR: Quantitative polymerase chain reaction

RDR6: RNA-dependent RNA polymerase 6

RISC: RNA-induced silencing complex

RNAi: RNA interference

ROS: Reactive oxygen species

rRNA: Ribosomal RNA

SA: Salicylic acid

SAR: Systemic acquired resistance

SAUR: Small auxin upregulated RNA

SGS3: Suppressor of gene silencing 3

siRNA: Small interfering RNA

SPCSV: Sweet potato chlorotic stunt virus

SPFMV: Sweet potato feathery mottle virus

SPLCV: Sweet potato leaf curl virus

SPPV: Sweet potato pakakuy virus

SPV2: Sweet potato virus 2

SPVC: Sweet potato virus C

SPVG: Sweet potato virus G

TGS: Transcriptional gene silencing

TJ: Tio Joe sweet potato cultivar

Trt – Treatment

vsiRNA: Viral small interfering RNA

WPI: Weeks post inoculation

1. INTRODUCTION

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; Luo et al., 2021). It can be eaten raw, steamed, baked or prepared as sweet potato pie, soup and fries. Sweet potato puree and flour can be used to make bread, cookies, cakes, or fermented to produce alcohol. Sweet potato is a good cover crop because it can spread its vines and cover the soil around it. Due to its ability to grow with minimal rain or irrigation, and the possibility to store the roots for an extended period, sweet potato is an essential crop for ensuring food and nutrition security, especially in Africa, where it's mainly grown for subsistence (Loebenstein and Thottappilly, 2009; Khoo et al., 2017; Low et al., 2017; Kreuze et al., 2020; CIP, 2022)

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. European production increased by 40.1%, accounting for 0.1% of global sweet potatoes in the same period (FAO, 2022).

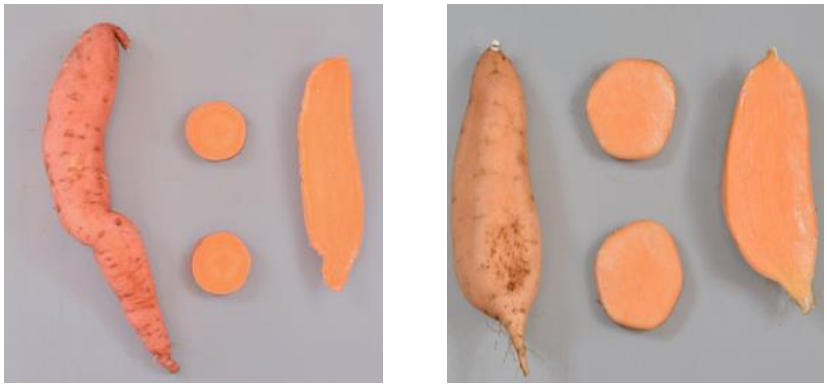
Globally, over 30 viruses affect sweet potatoes (Untiveros et al., 2007; Clark et al., 2012; Liu et al., 2020). It is critical that the propagation materials are free of viruses to avoid losses in yields and farmers' profits. Viral symptoms vary with the genotype, plant age, and environment. Some nutritional deficiencies can also be mistaken for viral symptoms (O'Sullivan et al., 1997; Gibson et al., 1998).

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. Transgenic sweet potatoes have been developed especially for resistance to SPFMV and SPCSV. Some transgenic plants were made to express parts of viral coat protein or rice cysteine proteinase inhibitor. However, the acquired resistance was only successful in greenhouses or against SPFMV (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). Resistance breeding should be broad regarding target virus strains, as resistance to a specific strain or variant of a virus may not hold for other strains or variants. SPCSV resistance was broken in cultivars resistant in West Africa for SPCSV-WA when grown in East Africa, where SPCSV-EA dominates

(Gibson et al., 1998). Conventional resistance breeding is difficult due to the self-incompatibility, heterozygosity, and hexaploidy of sweet potato (Loebenstein and Thottappilly, 2009). Understanding how the viruses infect and co-exist in the plant will enhance the development of solutions to control them. In the meantime, setting up a scheme and enacting laws to govern the production and use of virus-free sweet potato propagation materials will go a long way to salvage the current situation of sweet potato viruses in Hungary. This study sought to identify viruses infecting sweet potatoes in Hungary and attempt to remove them from cultivars preferred by farmers.

In addition to virus identification and elimination, this study sought to unravel the molecular mechanism of resistance to SPPV-SPCSV co-infection in sweet potatoes. Cuellar et al., (2009) suggested that viral suppressors of RNA interference (RNAi) possibly complement each other in sweet potatoes. Sweet potato - SPPV relationship seems symbiotic; the plant allows the virus to invade the meristematic and reproductive tissues, while the virus doesn't cause much damage to the plant and could even be beneficial to it (Roossinck, 2008, 2012; Bhat et al., 2016; Kreuze et al., 2020). We will conduct high throughput sequencing (HTS) and qPCR analysis of transcriptomes to confirm the interaction mechanism between sweet potato, SPCSV and SPPV in resistant and susceptible sweet potatoes (Kamitani et al., 2016; Maliogka et al., 2018).

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 (Figure 1), which are grown by farmers in Mozambique commercially and for subsistence (Musembi et al., 2019).



Tio Joe Melinda

Source: Musembi et al., (2019)

Figure 1. Root shape, skin and flesh colour of Tio Joe and Melinda cultivars grown by farmers in Mozambique. They have high beta-carotene content, are drought tolerant and resistant to sweet potato virus disease. Tio Joe was later found to be resistant to SPPV-SPCSV co-infection, while Melinda was susceptible.

An increase in production of these sweet potatoes will enhance the livelihoods of smallholder farmers and reduce pressure on other staples like rice, potato, maize, and wheat. Our findings will help breed disease-resistant sweet potatoes and develop sustainable control strategies (Pink and Hand, 2018).

2. OBJECTIVES

2.1 To detect viruses infecting sweet potatoes in Hungary.

Sweet potato was introduced in Hungary at least four decades ago. Its commercial production has risen in the last decade due to increasing consumer demand in the country. The storage roots are primarily used as food or for production of industrial starch, while the vines are used as animal feed. There is no record of landrace or indigenous Hungarian sweet potatoes. 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. Detected viruses will be sequenced to confirm their identities. Knowledge of the presence and distribution of the viruses will be valuable to the breeders, researchers and policy makers. Already four viruses have been reported in the country; therefore, it is imperative to have measures to control their spread.

2.2 To eliminate viruses from local sweet potato cultivars in Hungary.

Over one million sweet potato seedlings are produced annually in Hungary. The seedlings planted must be virus-free because viral diseases cause severe losses to farmers. There is no evidence of regulation on virus-free sweet potato production or planting in Hungary. This poses a phytosanitary risk due to the spread of the viruses across territories, especially in the European Union, and by aphids and whiteflies vectors in the fields. Heat treatment and meristem tip culture will be employed to remove viruses from selected farmers-preferred sweet potato cultivars in Hungary. Hopefully, this will create ground for establishing a program for producing pathogen-tested (PT) sweet potatoes for Hungary and the surrounding sweet potato growing regions.

2.3 Transcriptome analysis to elucidate the mechanism of resistance and susceptibility to SPPV-SPCSV co-infection.

SPPV is widespread and recalcitrant to remove, while SPCSV is the most damaging virus in sweet potatoes. Alone, the viruses do not cause much damage. We found a plant in a farmer's field in Hungary with severe disease symptoms yet contained only SPPV and SPCSV. Therefore, we sought to understand how the plant succumbed to the viruses. Pathogen-tested sweet potatoes will be graft-inoculated with SPPV-SPCSV to find resistant and susceptible cultivars to the dual virus infection. Total RNA isolated from a resistant and susceptible plant will be deeply sequenced, and the transcriptomes analysed to elucidate the mechanism of resistance and susceptibility and identify putative disease resistance genes for the viral co-infection. Knowledge of the resistance mechanism and putative disease resistance genes will help in breeding sweet potatoes resistant to the two viruses.

3. LITERATURE REVIEW

3.1. **Planting sweet potatoes**

Sweet potato belongs to the morning glory group of plants. It originated from the Americas around Peru and Ecuador between 6000-8000 BC (Loebenstein and Thottappilly, 2009; Clark et al., 2012). Since then, it spread to its secondary centres of diversity, including Asia, Africa and Europe in the 16th century. It is primarily grown in the tropics, mainly in East Asia and Sub-Saharan Africa, where it plays a significant role in food and nutrition security (O'Sullivan et al., 1997; Loebenstein and Thottappilly, 2009; Low et al., 2017).

Sweet potato grows well at low and high altitudes and requires 750-1000 mm rainfall, soil pH of 6.0, 24 ^{o}C temperatures and well drained loose soils for optimal growth. It can tolerate higher temperatures but not frost. Sweet potato planting materials are root sprouts or vine cuttings, which should be 30 – 40 cm long. The production of the planting materials should start in spring or at least three months before planting season. Lower and middle vine sections near the ground should be avoided as they could contain weevils. Young shoots are preferable as they are actively growing and could be virus free. Keeping cut vines and sprouts in a moist place for a day helps them overcome planting stress as roots develop. Three to five nodes should be put at least 5 cm deep in the soil during planting. Planting should be done on mounds of about 80 x 30 cm or 30 – 60 cm ridges with 100 cm spacing between them (Wilson, 1988; Gibson et al., 2009; Dennien et al., 2013; Muimba-Kankolongo, 2018). Watering in the first two weeks after planting is crucial to establish roots and shoots. Weeding is required until around two months after planting when leaves spread and cover the area around the plant.

The vines and leaves protect the soil from moisture loss and impede growth of weeds; therefore, it is a good cover crop. Harvested roots can be stored at 15^{0}C over winter without losing their viability and nutrients (Gibson et al., 1998; Loebenstein and Thottappilly, 2009; Muimba-Kankolongo, 2018). Sweet potato flesh and skin range in colour from white, cream, yellow, orange and purple based on their polyphenols content (O'Sullivan et al., 1997; Luo et al., 2021). According to FAO (2022), sweet potato was cultivated on 7.8 million hectares of land, which produced 91.8 million tonnes globally in 2019. China was the biggest producer, with an average production of 56 million tonnes from 2010 to 2019, followed by Malawi with 4.6 million tonnes.

3.2. **Sweet potato production in Hungary**

Sweet potato is only grown in Hungary from late spring to early autumn when the soils are warm. Production in Hungary is limited for reasons such as the unavailability of virus-free propagation materials. An increase in pathogens of sweet potato was predicted by Monostori et al., (2015) due to the increasing cultivation of the crop in Hungary. Spain and Portugal, the leading producers of

sweet potatoes in Europe, have warm temperate and Mediterranean climate conditions (Loebenstein, 2012). Although Hungary is in the temperate climatic zone (45°45'N and 48°35'N latitudes), it has a Mediterranean summer climate that favours sweet potato growth (Hungarian Meteorological Service, 2022). Sweet potato has been grown in Hungary for the last four decades, and its popularity is increasing among farmers and consumers. There is no system to provide farmers with PT propagation materials in Hungary. Most sweet potato farmers store harvested roots for production of sprouts for the next season planting or purchase sprouts from commercial producers (Monostori and Szarvas, 2015). When infected roots are used, this practice leads to persistence and accumulation of viruses in the crop. Four viruses were reported in sweet potatoes in Hungary (Salamon et al., 2020).

3.3. **Viruses of sweet potatoes**

Propagation of virus-infected vines and roots lowers yield and farmers' incomes. Besides lowering yields, viruses like SPFMV may cause internal corks and russet cracks, making the sweet potato roots unmarketable (Untiveros et al., 2007; Loebenstein and Thottappilly, 2009). Leaf yellowing, purpling, curling, rugosity, chlorotic spots, vein clearing, vein banding, stunting, leaf distortion and abaxial or adaxial swelling are the most common symptoms induced by viruses in sweet potatoes. These symptoms vary with cultivar, age of the plants and environmental conditions. Some viral infections can be symptomless, making it hard to recognize the infected plants visually (Gibson et al., 1997; Untiveros et al., 2007; Rukarwa et al., 2011; Clark et al., 2012; Loebenstein, 2012; Bhat et al., 2016; Liu et al., 2020). In addition, nutritional deficiencies often lead to symptoms that mimic viral infections (O'Sullivan et al., 1997; Gibson et al., 1998). Therefore, it is essential to verify the presence of viruses using robust, affordable and reliable diagnostic techniques.

Sweet potato is peculiar because up to eleven viruses can co-exist in a single plant with few adverse effects (Kwak et al., 2014; Liu et al., 2020). Most of the viruses are transmitted clonally through propagation materials, which exacerbate their spread. Mixed infections encourage recombination of viruses that may produce new variants, strains, or species, as in sweepoviruses (Liu et al., 2017). Wild relatives of sweet potatoes, such as *Calystegia hederacea* and *Ipomoea trifida,* act as reservoirs for viruses that affect the crop (Kreuze et al., 2020; Liu et al., 2021).

The most common viruses of sweet potato are sweet potato feathery mottle virus (SPFMV, family: Potyviridae, genus: *Potyvirus*) and sweet potato pakakuy virus (SPPV, Family: Caulimoviridae, Genus: *Badnavirus*). The economic importance of single virus infections in sweet potatoes is usually low, even in the case of sweet potato chlorotic stunt virus (SPCSV, family: Closteroviridae, genus: *Crinivirus*), which is quarantined in the European Union (EPPO, 2023).

SPCSV is the most important pathogen of sweet potatoes globally. It is limited to the phloem and is semi-persistently transmitted by whiteflies *Bemisia tabaci* and *Trialeurodes abutilonea* (Mukasa et al., 2006; Untiveros et al., 2007). The genome of SPCSV consists of two positive single stranded linear RNAs (+ssRNA) between 8.1-9.4 kb in length. RNA1 contains RNase3 and p22 (only East African strain) genes which can suppress gene silencing in plants (Altschul et al., 1990; Kreuze et al., 2002, 2005; Cuellar et al., 2009). Alone, SPCSV causes mild chlorosis and stunting. However, severe disease occurs when other RNA or DNA viruses synergize with SPCSV, leading to severe yield losses (Gibson et al., 1998; Untiveros et al., 2007). In complex infections with other viruses such as SPFMV and SPLCV, SPCSV synergizes with the viruses and increases their titres while its titre remains the same or even reduces. Co-infection of SPCSV and SPFMV causes the most severe sweet potato virus disease (SPVD), which may lead to 100% yield loss (Mukasa et al., 2006; Qin et al., 2014). Controlling SPCSV alone would significantly reduce the damage and economic loss brought about by sweet potato virus disease (SPVD). Plants infected with SPVD show chlorosis, are severely stunted, possess small, deformed leaves and give meagre yields (Aritua et al., 1998). Development of resistant cultivars through conventional breeding and genetic engineering has been successful either in greenhouses or against SPFMV only. The resistance in transgenic plants developed in different laboratories worldwide could not stand against the small and variable dosages of viral inoculations by vectors in the field (Clark et al., 2012; Sivparsad and Gubba, 2014). CRISPR-Cas gene editing has the potential to develop resistance to sweet potato viruses. Improved resistance to SPVD was observed in transgenic plants expressing an SPCSV-RNase3 targeted CRISPR- Cas13 vector (Yu et al., 2022).

Sweet potato pakakuy virus (formerly sweet potato badnavirus A and sweet potato badnavirus B), one of the most widespread DNA viruses in sweet potato germplasm globally, is transmitted by seeds and possibly by aphids or mealybugs vectors. It is a non-enveloped, bacilliform virus consisting of a capsid and an 8.1 kb circular double stranded (dsDNA) with four open reading frames. Some badnaviruses are integrated into the plant genome, but SPPV is not. According to Kreuze et al., (2020): (a) SPPV is not an endogenous virus, it is located in the cytoplasm as an episome in very low titres, and its variants are not mutually exclusive; (b) Transmission of SPPV by grafting to sweet potatoes or *Ipomoea setosa* is problematic, especially in the presence of SPFMV or SPCSV; (c) Co-infection with SPFMV or SPCSV slightly increased SPPV concentration; and (d) SPPV is not eliminated by heat treatment. The symptomless infection of badnaviruses allows them to spread passively through propagation and sharing of infected planting materials. The passive transmission of SPPV poses a serious phytosanitary challenge to the international movement of sweet potato germplasm, especially since it is not easily removed by

chemical and heat therapies (Varveri et al., 2015; Bhat et al., 2016; Kreuze et al., 2020; Kiemo et al., 2022b).

Knowledge of the presence and distribution of viruses is essential for their control. Understanding the molecular mechanism of infection of SPCSV and other viruses, especially SPFMV and SPPV, will help provide a baseline for the development and breeding of virus resistant sweet potatoes (Clark et al., 2012; Gibson and Kreuze, 2015). This can be achieved through genomic and transcriptome analysis of infected, PT, resistant, and susceptible sweet potatoes (Bednarek et al., 2021).

Potyviruses SPFMV, SPVG, SPVC and SPV2 are non-persistently spread by aphids *Aphis gossypii* and *Myzus persicae* in sweet potatoes. They are approximately 850 nm long flexuous particles with a +ssRNA genome of about 10.8 kb. The potyviruses are so closely related that SPVC was once referred to as SPFMV common (C) strain. SPFMV, whose typical foliar symptom is feathery mottling, has russet crack, ordinary and East African strains. Genome recombination occurred in potyviruses complex infections, given the high similarity of their coat protein sequences. The viruses are mostly symptomless in single infections, but during complex infections with other viruses, they disrupt photosynthesis, resulting in chlorotic spots and yield loss. The potyviruses cause chlorosis, mottling and vein clearing in *I. setosa.* Severe disease occurs in complex infections involving potyviruses and SPCSV. (Ateka et al., 2004; Clark and Hoy, 2006; Kreuze and Fuentes, 2008; Shi et al., 2019). In Europe, SPCSV, SPFMV, SPVG and SPV2 have been reported in Spain (Trenado et al., 2007) and SPCSV, SPFMV, SPVC and SPV2 in Portugal (Varanda et al., 2015; EPPO, 2023).

SPLCV (Family: Geminiviridae, Genus: *Begomovirus*) exists as twin icosahedral particles with a monopartite circular ssDNA genome of about 3 kb (Cho et al., 2020). Frequent recombination events have produced multiple strains and variants of sweet potato begomoviruses, collectively named ‘sweepoviruses’ (Clark et al., 2012). SPLCV causes yield loss of up to 60% (Ling et al., 2010). It is horizontally transmitted by whitefly *Bemisia tabaci* and vertically through seeds (Clark et al., 2012). Its typical symptom is upward curling of leaves in *I. batatas* and *I. setosa*. The symptom disappears in sweet potatoes as they mature, but the virus remains. Synergy with other viruses, such as SPCSV, increases SPLCV titres without increasing yield loss (Ling et al., 2010; Wanjala et al., 2020).

3.4. **Virus infection severity**

Successful RNAi reduces virus titres, which can be associated with mild or lack of disease symptoms in the young leaves of resistant cultivars (Soosaar et al., 2005). In susceptible cultivars, however, RNAi is insufficient to prevent virus accumulation leading to increased virus titres and related symptoms, which may be lethal (Kreuze et al., 2008; Yu et al., 2022).

Symptoms develop when viruses interfere with the expression of host genes for physiological processes such as cell division, growth, defence, photosynthesis, protein synthesis, respiration and transport (Havelda et al., 2008; Tu et al., 2015). Development of symptoms is a function of the interaction between the host and pathogen and may differ even in plants of the same cultivar. It may also be influenced by abiotic factors such as temperature, drought and humidity. Some viral infections are not accompanied by any symptoms. Symptoms, therefore, are not always a true reflection of the severity of viral infection in sweet potatoes (Gibson et al., 1998; Szittya et al., 2003; Havelda et al., 2008; Burgyán and Havelda, 2011).

A plant is resistant to disease or pathogen when it can be infected by the pathogen but prevents spread of the pathogen. Resistance can be classified as high or intermediate, depending on the severity of the symptoms (Mwanga et al., 2013). A resistant plant may show mild, delayed or no symptoms at all. A susceptible plant, however, will not limit the spread, multiplication and effect of the pathogen; therefore, it can develop severe symptoms early during the infection period, depending on the virulence of the pathogen and the growth conditions. Concentration or titre of a virus might also affect the magnitude of disease. A higher virus concentration usually leads to more severe symptoms. Resistant plants often fight to keep the virus concentration low through systemic acquired resistance (SAR) and RNAi. The type and severity of symptoms expressed is often linked to the kind and intensity of infection. A more virulent virus often interferes with the plant's physiological and developmental processes causing disease symptoms (Loebenstein and Carr, 2006; Untiveros et al., 2007). Combined virus titre and symptom evaluation is a more reliable measure of pathogenicity and resistance, and would give a more accurate picture of viral disease severity (Tavantzis, 1984; Karyeija et al., 1998; Loebenstein and Carr, 2006; Miano et al., 2008; Mwanga et al., 2013).

3.5. **Virus diagnosis**

Current virus detection methods include molecular tests (PCR and qPCR or RT-PCR), enzyme-linked immunosorbent assay (ELISA) and bioassays. *I. setosa* is an excellent indicator plant for sweet potato viruses due to its sensitivity to the viruses, which are more evenly distributed and in higher titres making it easier to detect them than in sweet potatoes (Kokkinos and Clark, 2006b; Rukarwa et al., 2011). PCR and RT-PCR are very sensitive, fast and reliable virus testing methods. However, they are limited because they need knowledge of primer sequences; therefore, they may not detect unknown viruses present in a sample. These techniques have detected several RNA and DNA viruses in sweet potatoes. However, there could be more undetected viruses which could be important for farmers, hence the need for HTS (Kreuze et al., 2009). HTS can detect novel viruses even in extremely low titres, but it is not used for routine tests due to its high cost and complexity

(Kreuze et al., 2009; Maliogka et al., 2018). In RNA sequencing (RNA-Seq), millions of RNA reads are generated within a short time from a sample, providing big data which can be used to explain changes in transcriptome during virus infection at a given time (Kamitani et al., 2016). A portable tool developed to test for SPFMV, SPCSV and SPLCV using loop-mediated isothermal amplification (LAMP) in the field will help speed up diagnosis and save costs (Wanjala et al., 2021).

3.6. **Virus elimination**

Preventing the introduction and spread of virus infected germplasm is crucial to avoid epidemics (Gibson et al., 1998). Production of clean virus-free or PT vines is the most used control measure to date (Beetham and Mason, 1992). However, re-infection occurs when virus-free plants are planted near infected ones. This is common, especially for asymptomatic viruses like SPFMV, SPLCV and SPPV, because farmers cannot visually discriminate between the infected and healthy vines.

Plants can recover from virus infection in high temperatures (Ssamula et al., 2020). The heat slows down the multiplication and spread of viruses through increased RNAi or by inhibiting virus replication and movement through SAR (Ryals et al., 1994; Szittya et al., 2003; Soosaar et al., 2005; Rukarwa et al., 2011).

Presence of low virus titre in a plant is a characteristic of recovery from virus infection, whereby the plant inhibits systemic virus spread but doesn't eliminate the virus particles. Recovery from viruses can be seen when the new young leaves of an infected plant are symptomless. However, the virus may still be detected in them, so the plant is said to be tolerant or resistant. Recovery from viral diseases, including SPVD, occurs in the fields in East Africa due to the high tropical temperatures. Propagation of the recovered shoots by small holder farmers helps overcome cultivar degeneration and preserve yielding (Tavantzis, 1984; Mwanga et al., 2013; Gibson and Kreuze, 2015; Kørner et al., 2018; Paudel and Sanfaçon, 2018; Bradamante et al., 2021).

Cell division in the meristems is often faster than viruses can replicate and infect the new cells. Consequently, some meristematic cells may be virus-free (Rukarwa et al., 2011; Varveri et al., 2015; Kidulile et al., 2018; Wang et al., 2018). The meristematic tip can then be carefully cut out and cultured in a media to produce plants, which should be tested to confirm their virus-free status (Beetham and Mason, 1992; Rukarwa et al., 2011; Dennien et al., 2013). In sweet potatoes, the success of virus elimination by heat therapy varies with genotype, treatment plant and viruses present. Single viruses and micropropagated plants are easier to clean than multiple infections and potted plants (Rukarwa et al., 2011).

3.7. **Plant defence against viruses**

Timely recognition and transduction of pathogen invasion signals coupled with speedy deployment of immune responses is critical to virus resistance (Paudel and Sanfaçon, 2018). Plants have receptors (virulence factors), which recognize effectors (avirulence factors). The avirulence factors are proteins encoded by viruses and are essential for initiating an attack in their host. The plant basal defence system triggers defence responses after recognizing pathogen avirulence factors either directly by interacting with them in a gene-for-gene fashion or through their effects on the host metabolic processes (Flor, 1971; Pumplin and Voinnet, 2013). Recognition of virus avirulence factors like double stranded RNA (dsRNA) intermediates, viral mRNA or viral proteins trigger defence signal transduction, leading to cellular processes, particularly RNAi and SAR, that prevent or limit the spread of infection.

Although disease resistance (R) genes encode proteins which protect against pathogens in a gene-for-gene model (Flor, 1971; Baulcombe, 2004), resistance or susceptibility to a pathogen, may not be attributed to upregulation or downregulation of a particular gene per se. Nevertheless, some gene(s) may profoundly impact plant-pathogen compatibility. Recessive resistance may be attributed to mutation or inhibition of host cofactors critical to virus infection and replication, such as eukaryotic initiation factor 4E protein (eIF4E) (Baulcombe, 2004; Nicaise, 2014; Hashimoto et al., 2016; Machado et al., 2017; Paudel and Sanfaçon, 2018).

3.7.1. **RNA interference**

The plant defence mechanism through dicer-like enzymes (DCLs) recognizes and binds to dsRNA intermediates of viruses in the cytoplasm triggering RNAi or gene silencing, which leads to cleavage of the viral nucleotides and suppression of infection. The process of RNAi against viruses is as follows: DCL2 and DCL4 initiate post-transcriptional gene silencing (PTGS) against RNA viruses by binding to and cleaving viral dsRNA intermediates to 21-22 nt fragments known as viral small interfering RNAs (vsiRNAs). DCL3 and DCL4 initiate transcriptional gene silencing (TGS) against DNA viruses. DCL3 cleaves dsRNA into 24 nt fragments. HUA ENHANCER 1 (HEN1, sRNA 2'-O methyltransferase) protects the 21- 24 nt vsiRNAs from degradation by methylating their 3′ end. The stabilized vsiRNAs are then loaded to argonaute (AGO) proteins. PTGS-derived vsiRNAs are loaded to AGO1, AGO2, AGO5 and AGO7, while TGS vsiRNA to AGO4. In PTGS, transcription factors such as WRKY3 and miR396 bind to loaded AGOs to form RNA-induced silencing complex (RISC). RISC targets complementary viral messenger RNA (mRNA) and cleaves them to produce more vsiRNA. RNA-dependent RNA polymerase 6 (RDR6), while interacting with the suppressor of gene silencing 3 (SGS3) and silencing defective 5 (SDE5) cofactors, synthesise more vsiRNA to amplify the RNAi. The vsiRNAs are then loaded to AGOs which target more complementary viral mRNAs for cleavage or methylation, thereby

suppressing virus accumulation and infection spread. In TGS, the loaded AGO4 leads to methylation of the viral DNA or histones, preventing its transcription (Baulcombe, 2004; Carr et al., 2010; Burgyán and Havelda, 2011; Pumplin and Voinnet, 2013; Csorba et al., 2015; Machado et al., 2017).

Most viruses, including SPCSV and SPFMV, encode proteins that suppress plant defence responses (Burgyán and Havelda, 2011; Pumplin and Voinnet, 2013; Csorba et al., 2015). For example, some potyviruses like sweet potato mild mottle virus (SPMMV) possess P1 RNA silencing suppressors, which bind to AGO1 through conserved WG/GW motifs (Clark et al., 2012; Csorba et al., 2015; Kenesi et al., 2021). SPCSV RNAse3 is a class I endonuclease that interferes with RNAi in sweet potatoes by cleaving dsRNA and 21-22 nt small interfering RNAs (siRNAs) to approximately 14 nt fragments which cannot be loaded to the AGOs during RISC formation (Cuellar et al., 2009; Weinheimer et al., 2016). The SPCSV RNAse3 also interacts with SGS3 and RDR6 to suppress production of siRNAs which amplify RNAi (Weinheimer et al., 2016). Since RNAi is one of the advanced innate defence mechanisms plants have against pathogens, its suppression seriously affects the plant's defence system, exposing it to severe diseases like SPVD (Burgyán and Havelda, 2011; Csorba et al., 2015). Complementarity or additive effects of different viral suppressors of RNA silencing, such as RNAse3, p22 and P1, could be responsible for the successful attacks (Cuellar et al., 2009).

3.7.2. **Systemic acquired resistance**

In SAR, phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are used in an innate immune response, which includes inducing hypersensitive response that leads to programmed cell death and necrosis at the point of infection, inhibiting spread of the virus. SAR leads to production of pathogenesis-related (PR) genes, biosynthesis of secondary metabolites, detoxification and cell wall strengthening to prevent pathogen spread from infected cells (Blée, 2002; Almagro et al., 2009). SAR also primes parts of the plant distant from the infection point for defence against the invading pathogen (Ryals et al., 1994; Soosaar et al., 2005; Carr et al., 2010; Zvereva and Pooggin, 2012; Kidwai et al., 2020).

SAR signalling is modulated by ROS levels in the cells and tissues. Reactive oxygen species (ROS) is found in plants as oxide (O^{-2}), hydroxide (OH^{-}) or hydrogen peroxide (H_2O_2). It is produced and utilized in the apoplast and cytosol by enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and type III peroxidase in response to SA and PR proteins signalling (Almagro et al., 2009). ROS, auxins (mostly indole-3-acetic acid - IAA), JA, SA and ET levels are cross-linked with SAR, RNAi and disease resistance (R) genes responses. ROS regulates transcription of defence genes in the nucleus. Vitamins such as thiamine, with

antioxidant and ROS-modulating activity, can also induce SAR. Interaction of the phytohormones and disease resistance genes balances growth and defence processes (Ahn et al., 2005; Zvereva and Pooggin, 2012; Boubakri et al., 2016; Iswari and Palta, 2018; Kidwai et al., 2020).

Viruses often trigger generic stress responses with no specific gene-for-gene resistance mechanism in plants (Whitham et al., 2006; Paudel and Sanfaçon, 2018). They include upregulation of several PR genes, including peroxidase, chitinase, glucanase and phytoalexins, which are involved in SAR response to virus infection (Saboki et al., 2011; Singh and Singh, 2018). Glucanases can produce carbohydrate elicitors against the invading pathogen, while phytoalexins have antimicrobial and antioxidant activity, which prevents pathogen accumulation. PR proteins also lead to increased callose deposition on the cell walls, making it difficult for viruses to spread from the infected cells. These PR proteins are released early during infection in resistant plants. Basic PR proteins are normally found in the vacuole, and their upregulation is enhanced by ET and methyl jasmonate during biotic stress (Xu et al., 1994; Saboki et al., 2011; Singh and Singh, 2018).

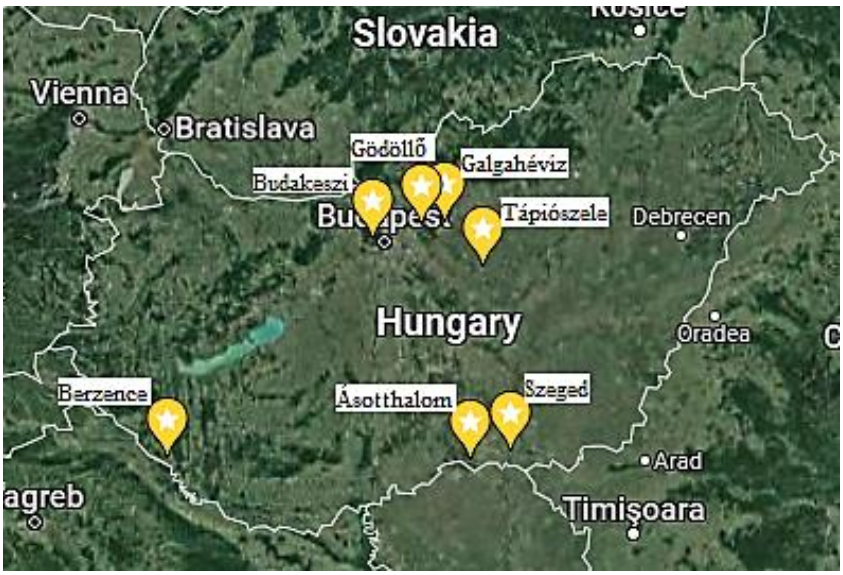
4. MATERIALS AND METHODS

A flow chart was constructed to illustrate interconnections between the experiments conducted (Appendix 12.4).

4.1. Sources of plant materials

4.1.1. Plant materials for detection of viruses infecting sweet potatoes in Hungary

In Hungary, we collected 62 symptomatic and 38 symptomless sweet potato vines from farmers' fields in Galgahévíz (n=25), Ásotthalom (n=70) and Szeged (n=5); and storage roots from researchers in Szeged (n=12) and Gödöllő (n=2); gene bank in Tápiószele (n=4); and retail stores in Budakeszi (n=4) and Berzence (n=6) between 2019 and 2021 (Figure 2).



(Source: Google maps)

Figure 2. Map of Hungary showing sample collection points

Some of the vines collected from farmers' fields in Hungary had apparent disease symptoms (Figure 3). All the samples collected were planted in soil in pots in an insect-proof greenhouse at the Institute of Genetics and Biotechnology of The Hungarian University of Agriculture and Life Sciences in Gödöllő, Hungary. Viral symptoms were evaluated in all the plants and recorded for 12 weeks (Table 2).

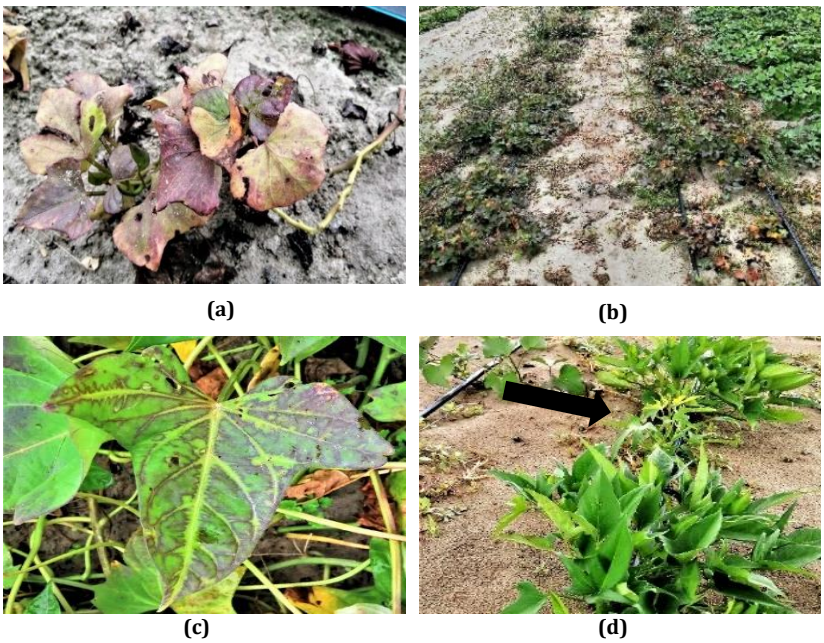


Figure 3. Virus infected sweet potatoes in the fields in Hungary. Severe stunting, leaf distortions and chlorosis symptoms characteristic of SPCSV complex infections (a and b); Feathery mottling typical of SPFMV (c); Stunting and chlorosis in a PT plant which got infected in the field (black arrow) between healthy plants of the same cultivar. It was later found to contain SPFMV, SPVG, SPLCV and SPPV (d).

4.1.2. **Plant materials for SPPV-SPCSV resistance screening**

Eighteen PT sweet potato cultivars were obtained as *in vitro* plantlets and cuttings in January 2020 from the International Potato Center (CIP) in Nairobi and shipped to the Institute of Genetics and Biotechnology of The Hungarian University of Agriculture and Life Sciences in Gödöllő, Hungary (Table 1). The cultivars were selected from the sweet potato catalogue based on their yielding capacity, maturity period, and resistance to sweet potato virus disease (SPVD) according to the 2014 and 2019 orange fleshed sweet potato catalogues (Tumwegamire et al., 2014; Musembi et al., 2019).

The PT sweet potatoes were micropropagated with PT *Blk* and *Ylw* cultivars from our Hungarian collection. Their progenies were transplanted to jiffy, then to soil and grown separately in pots for two months in an insect-proof greenhouse.

One plant collected from Ásotthalom, Hungary and labelled *A6.1* had severe disease symptoms in the greenhouse (Table 2). 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.

Table 1. Pathogen-tested sweet potato cultivars selected for resistance screening to sweet potato pakakuy virus and sweet potato chlorotic stunt virus co-infection

No.	Cultivar/Genotype	Sweet potato virus disease (SPVD) resistance	Maturity (months)
1.	Amelia	High	5
2.	Bela	High	5
3.	Cecilia	High	5
4.	**Irene (Kakamega 7)**	**High**	**5**
5.	**Melinda**	**High**	**5**
6.	**Ndamirabana (RW11-2910)**	**High**	**5**
7.	**Tio Joe**	**High**	**5**
8.	Kemb10	Moderate	4
9.	Kenspot 5	Moderate	5
10.	**Mugande**	**Moderate**	**4**
11.	NASPOT 10 O (Kabode)	Moderate	4
12.	Sumaia	Moderate	5
13.	**Ukerewe**	**Moderate**	**4**
14.	Cacearpedo	Susceptible	4
15.	**Ejumula**	**Susceptible**	**4**
16.	Kenspot 4	Susceptible	5
17.	**Mayai**	**Susceptible**	**4**
18.	**Sinia B**	**Susceptible**	**4**
19.	*Blk*	Unknown	4
20.	***Ylw***	**Unknown**	**4**

Note: Ten boldened cultivars were selected for a second test

4.2. **Molecular tests**

4.2.1. **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. The three leaves increased the chances of virus detection by PCR and qPCR.

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).

The leaf samples were collected using a well-labelled 1.5 ml Eppendorf tube containing glass beads for homogenization. ZenoGene Kit (Zenon Bio, Szeged, Hungary) was used to extract DNA. 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. Manufacturer's instructions for each kit were followed.

4.2.2. **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. To increase specificity, exon-spanning primers were preferred for gene expression analysis (Appendix 12.2).

4.2.3. **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 at 94 °C 4 min, 94 °C 1 min, 50–58 °C (depending on the annealing temperature of the primers) 30 sec, 72 °C 80 sec and 72 °C 10 min for 30 cycles. 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 (Appendix 12.2.1). 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 (94 ºC 4 min, 94 ºC 1 min, 55 ºC 30 s, 72 ºC 70 s and 72 ºC 10 min for a total of 30 cycles) using the same primers used for qPCR.

Sweet potato chlorotic stunt virus RNA1 and RNA2 complete genome sequences from sample *A6.1* were obtained via PCR amplifications of cDNA using primers (Appendix 12.2.2) designed (from acc. KC888966 for RNA1 and acc. KC888963 for RNA2) to amplify overlapping fragments of SPCSV West African strain.

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. Ten times diluted cDNA and SPPV_CP2 and CH2N primers (Appendix 12.2.1) were used for qPCR using the qPCRBIO SyGreen qPCR kit, 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 normalization to neutralise differences in sample quantities.

Primer amplification efficiency was calculated using the formula:

Amplification efficiency (%) = (10^(-1/slope)-1)*100.

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.

4.2.4. **Cloning and sequencing**

The amplified DNA fragments were purified using QIAquick gel extraction kit (QIAGEN, Hilden, Germany), cloned into pGEM-T Easy Vector (Promega, Madison, USA) and sequenced using Sanger sequencing method (Biomi, Gödöllő, Hungary).

GenBank BLASTn search confirmed the identity of each of the viruses detected.

4.3. **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. The vines were cut and propagated vegetatively to make 30 plants per cultivar. 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 25^0C for 7 days, 29^0C for 14 days and 39^0C for 28 days to free them of viruses (Dennien et al., 2013). Surviving shoot tips (2 cm) were cut out, washed and sterilized. 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 (Figure 11). The plantlets were multiplied *in vitro*, and progenies acclimatized 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.

4.4. **Bioassay**

To confirm the presence and infectivity of the viruses detected, scions from 50% of the virus infected sweet potatoes, according to PCR and qPCR tests, were grafted to *I. setosa* indicator

plants. Heat-treated plants which didn't have viruses (except SPPV) according to PCR and qPCR were also grafted to *I. setosa* to confirm their 'virus-free' status.

Wedge and side grafts were made to enhance virus transmission to the indicator plant (Figure 4). An *I. setosa* grafted with a virus-free sweet potato scion served as a negative control (Dennien et al., 2013). Symptoms were evaluated in *I. setosa* plants for eight weeks and recorded (Table 2, Figure 11). DNA and RNA were extracted from leaves of the grafted *I. setosa* to test for viruses by PCR and qPCR.

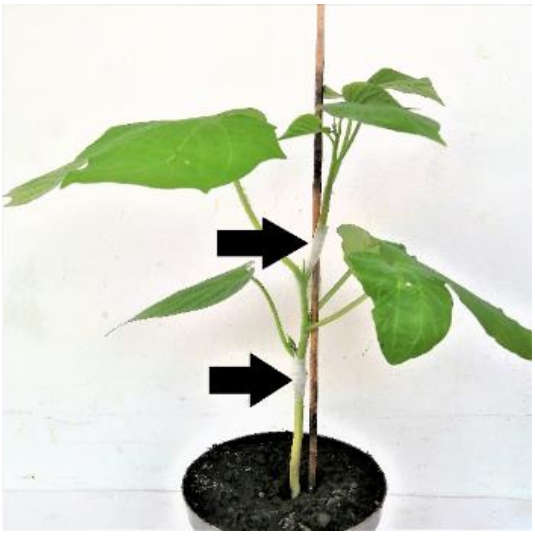


Figure 4. Wedge and side grafting of sweet potato scions to *I. setosa* rootstock. Arrows show the graft joints.

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

Test I: The eighteen *in vitro* PT plants from CIP and two PT cultivars from our Hungarian collection labelled *Ylw* and *Blk* were planted in pots after acclimatization from the *in vitro* chamber (Table 1). The SPPV-SPCSV infected *A6.1* was propagated for two months alongside the PT cultivars in the greenhouse before grafting. They were irrigated twice a week, and pesticides sprayed fortnightly.

Wedge grafting was done using the PT plants as scions and the SPPV-SPCSV infected *A6.1* as the rootstock. Control plants were made for each treatment whereby the scion of each cultivar was mock grafted to its rootstock. After grafting, the plants were covered with a transparent plastic bag to prevent moisture loss and put in darkness in a growth chamber for three days at 23 ^{o}C. They were then maintained in the growth chamber at 23 ^{o}C and 16 hours of light daily. Symptoms were evaluated weekly for twelve weeks. Leaf samples were collected from the top fully open leaf at the 3rd, 6th, 9th and 12th weeks post inoculation (wpi). The samples were dipped in liquid nitrogen and stored at -80 ^{o}C until when RNA was isolated and virus titres measured by qPCR.

Test II: Based on the result of the first test and resistance to SPVD according to the online sweet potato catalogues (Tumwegamire et al., 2014; Musembi et al., 2019), ten PT sweet potato cultivars were selected for further testing (boldened in Table 1).

To reduce variabilities related to age, vine vigour, and environmental conditions found in the greenhouse, such as temperature and lighting, *in vitro* propagated plants were used. About 2 cm cuttings were made from the tips of the infected *A6.1* vines. They were cleaned, sterilized and micropropagated *in vitro* in half-strength MS media as the PT plants. All the plants were carefully micropropagated under a sterilized laminar flow cabinet to avoid cross contamination until there were enough vines for three biological replicates plus controls.

The *in vitro* micropropagated plants were transferred to jiffy and acclimatized in a growth chamber at 23 ^{o}C, 16 hours light daily for two weeks. They were then potted in soil and grown under the same conditions for eight weeks. Wedge grafting was done for virus inoculation when the plants attained 6-8 nodes length. Three biological replicates with one control were wedge-grafted. Leaf samples were collected for DNA and RNA extraction in the 1st and 3rd wpi. For RNA isolation, the tubes were dipped in liquid nitrogen and then stored at -80 ^{o}C until when RNA was isolated and virus concentration estimated by qPCR.

4.6. **RNAi genes expression analysis**

To check differences in expression of genes involved in RNAi, primers were designed for DCL2, DCL4, AGO1, AGO4 and SDE5 (Appendix 12.2.3). Gene expression analysis was carried out in Melinda and Tio Joe cultivars by qPCR in three biological replicates with two technical replicates and actin as a reference gene using the delt-delta Cq method.

4.7. **Transcriptome analysis**

Based on the results of the first and second tests Melinda and Tio Joe cultivars were selected for transcriptome analysis to investigate the resistance and susceptibility mechanism in SPPV-SPCSV co-infection. Two treated biological replicates of each cultivar, which showed similar phenotypic (symptoms) and genotypic (gene expression, virus accumulation) characteristics, were selected for HTS; these were Melinda replicates labelled b and c, and Tio Joe replicates labelled a and c.

4.7.1. **Library preparation and deep sequencing**

Total RNA from the two treated biological replicates was pooled to enrich the transcriptome. Four treatments were made based on the cultivar and period of RNA collection. These were labelled ME_1, ME_3, TJ_1 and TJ_3, where ME – Melinda, TJ – Tio Joe, 1,3 – wpi. RNA was prepared for HTS (IbioScience, Pécs, Hungary) from the four treatments and their mocks in the ratio of 1:1 treatment to mock.

The library for Illumina sequencing was prepared using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, Ipswitch, MA, USA). The ribosomal RNA (rRNA) was removed from 400 ng total RNA using RiboCop rRNA depletion kits (Lexogen, Ipswitch, Austria). After that, the rRNA depleted RNA was fragmented, end-prepped, and adapter-ligated. Finally, the library was amplified according to the manufacturer's instructions. The quality of the libraries was checked on Agilent 4200 TapeSation System using D1000 Screen Tape (Agilent Technologies, Palo Alto, CA, USA). The quantity was measured on Qubit 3.0. Illumina sequencing was performed on the NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) with a 2 ×151 run configuration.

The transcriptomes used in this study were deposited in the NCBI GenBank sequence read archive (SRA) under the BioProject accession number: PRJNA973635.

4.7.2. **Reads assembly and mapping**

FASTQ files containing the raw HTS data were imported as paired reads to CLC Genomics Workbench v 21.0.5 (QIAGEN, Aarhus, Denmark) for analysis. Trimming was done at a quality score limit of Q = 0.05. Ambiguous nucleotides, adapters and homopolymers of A, C, G and T nucleotides were removed from the 5' and 3' ends. Reads shorter than 15 bp were discarded. Trimmed reads were mapped using default settings (length fraction = 0,5 and similarity fraction = 0,8) to *Ipomoea trifida* genome (NSP306, Hard Masked Genome Assembly, v3) (Wu et al., 2018). Unmapped reads were collected to check for viruses.

4.7.3. **De Novo assembly and virus search**

Unmapped reads were de novo assembled into contigs in CLC. A BLAST database of all viruses reference sequences (https://ftp.ncbi.nlm.nih.gov/refseq/release/viral/viral.1.1.genomic.fna.gz) was downloaded on 23 December 2022 from the NCBI GenBank. The database was used as a reference to search for viruses present in the assembled contigs using the BLASTn program with an expectation value of 1.0E-50. Reference sequences for the best hits were downloaded from GenBank and aligned to the unmapped reads to check their presence and expression level in the mock and treated samples.

4.7.4. **RNA-Seq and differential gene expression analysis**

Working model tracks from *I. trifida* hard-masked genome, coding sequences (CDS), gene and mRNA (Wu et al., 2018) were used as references for gene expression analysis in CLC. The trimmed reads were mapped to the reference tracks using default settings (length fraction = 0.8, similarity fraction = 0.8), and gene expression values calculated as transcripts per million (TPM) reads. Differential gene expression (DGE) analysis for the whole transcriptome was done by

comparing treatments to their respective mocks. Volcano plots of up and down regulated genes were created.

4.7.5. Correlation analysis

Principal Component Analysis (PCA) for all samples was performed in CLC to check variability within the dataset. A heat map was constructed using Euclidean distance and complete linkage clustering. A Venn diagram was created to check the number of overlapping differentially expressed genes.

4.7.6. Functional annotation and gene set enrichment analysis

OmicsBox Blast2Go software (Götz et al., 2008) was used for functional analysis and gene annotation of 60,116 coding sequences (NSP306_trifida_v3.working.gene_models.pep.fa) of *I. trifida* (Wu et al., 2018). Further analysis of the 35,042 annotated features obtained from Blast2Go was performed in CLC and Database for Annotation, Visualization and Integrated Discovery (DAVID) online software (v Dec. 2021) using default settings (Huang et al., 2009; Sherman et al., 2022).

In CLC, differentially expressed genes (DEGs) with mean expression values above 5.0, absolute log2 fold change ≥ 1.5 and false discovery rate (FDR) p-value of ≤ 0.05 were selected for gene set enrichment analysis. Expression browsers containing the gene and transcript expression, statistical comparisons (between the mocks and treatments) and gene set enrichment analysis data were created and exported to Ms Excel for further analysis.

Homologues of *I. trifida* coding sequences from *Arabidopsis thaliana* were obtained through NCBI BLASTp and used to carry out additional functional analysis in DAVID. Only DEGs with a fold change ≥ 1.5 and p-value ≤ 0.05 were used in DAVID, and results exported to Ms Excel for further analysis.

4.7.7. qPCR validation of RNA-Seq results

Primers were designed for six randomly selected DEGs to validate their expression by qPCR (Appendix 12.2.3). Primer design, calculation of their amplification efficiency and qPCR were performed as described above. qPCR was performed 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 normalization, and relative gene expression was calculated using the delta-delta Cq method.

5. RESULTS

5.1. Virus symptoms in *I. batatas* and *I. setosa*

Symptomatological surveys revealed that 30 to 80% of sweet potato plants in the fields showed viral symptoms like stunting, leaf curling, mottling, purpling and yellowing, which were also observed in the transplants grown in the greenhouse (Table 2). The symptoms mostly appeared in young leaves four weeks after planting, changed gradually and disappeared 2-3 weeks later in some plants as the symptomatic leaves grew older. Viral symptoms in sweet potatoes included vein clearing, leaf and vein chlorosis, feathery mottling, chlorotic spots, leaf distortion, leaf purpling, rugosity, chlorosis, adaxial swelling, puckering and stunting. Chlorosis and chlorotic spots were the most common, as is characteristic of potyviruses. Plants infected with SPCSV displayed more severe symptoms, although the severity of the symptoms was not correlated with the number of viruses. Plants infected with DNA viruses SPPV and SPLCV were asymptomatic and only developed mild symptoms occasionally.

I. setosa developed symptoms within three wpi, which included leaf and vein chlorosis, leaf distortion, vein clearing, mottling and stunting. Severe stunting and chlorosis occurred in SPCSV infected *I. setosa*, most of which didn't survive past three wpi.

Table 2. Viruses detected in *I. batatas* and *I. setosa* and their symptoms in the greenhouse

		Molecular test							No. of viruses		Weeks after planting	Weeks post inoculation	Symptoms	
		qPCR					PCR							
No.	Sample ID	SPFMV	SPVG	SPVC	SPV2	SPCSV	SPLCV	SPPV	*I. batatas*	*I. setosa*	*I. batatas*	*I. setosa*	*I. batatas*	*I. setosa*
1	22C	-/+	-/+		.		-/+	+/+	1	4	2	2		
2	48C	-/+					+/+		1	2	9	2		
3	30C	+/+						+/-	2	1				
4	3C	+						+	2					
5	37C		-/+	+/+	+/+		+/+	+/-	4	4				

6	Gdl_a			+					1					
7	Gdl_b							+	1					
8	Gz1.2	+/+		+/+			+/+	+/-	4	3				
9	Gz2.2	+/+		+/+	+/+			+/-	4	3	11	2		
10	Gz2.3	+/+	+/-		+/-			+/-	4	1	6	3		
11	A2v1	+/+							1	1	6	2		
12	A3v3							+	1					
13	A3v4		+						1		6			
14	A3v5							+	1					
15	A6v10	+/-				-/+			1	1				
16	3 n_Af1		+						1					
17	5 n_Af1		+						1					
18	6 n_Af1		+						1					
19	A4w1	+							1					
20	A4Y1							+	1					
21	A7.1							+	1					
22	A6.1					+/+		+/-	2	1	9	2		
23	A6v9 -2	-/+			-/+	+/+		+/-	2	3	4			
24	4 n_Af1	+	+						2					
25	Ylw	+		+					2					
26	A4w2			+				+	2		7			
27	A4Y2				+			+	2					

28	A6v7	+		+					2					
29	A6v8	+		+					2					
30	A6v9 -1	-/+			+/+	+/+		+/-	3	3	3	3		
31	A1Y1	+					+	+	3					
32	A2v2	+		+				+	3					
33	A5.1				+	+		+	3					
34	A5v6	+		+				+	3					
35	A7v11	+/+	+/+	+/+				+/-	4	3				
36	Blk	+		+	+			+	4		7			
37	A3Y1	+	+		+			+	4					
38	A2Y1	+	+	+	+		+	+	6		7	2		
39	A7	-/+						+/-	1	1				
40	Bat 2				+				1					
41	Bat 3	+/+						+/-	2	1	6	3		
42	A1			+				+	2					
43	A10	-/+	+/+	+/+			+/+	+/-	3	4	8	2		
44	A6	-/+	+/-	+/-				+/-	3	1	8	2		
45	A3	-/+	+/+	+/+	-/+			+/+	3	5				
46	A5	+/+	+/+	-/+	-/+			+/-	3	4				
47	A8	+/+	+/+	+/+	-/+				3	4				
48	A4			+	+			+	3					
49	A9	+		+				+	3		8			

50	A2	+/+	+/+	+/+	-/+			+/+	4	5				
51	T96	-/+	-/+	-/+	-/+			+/-	1	4	9	2		
52	12R						+/+	+/-	2	1	5			
53	105R	+/+	-/+	+/+	-/+				2	4	8			
54	92R	+/+	+/+	+/+	+/+			-/+	4	5	9	2		
55	USBdz							+	1		5			
56	USBer 1	+/+				+/+		+/-	3	2				
57	USBer 2	+/+				+/+		+/-	3	2				
58	USBer 3	+/+		+/+	+/+	+/+	+/+	+/-	6	5	7			
59	USBer 4				+				1					

Note: ±/± virus status in *I. batatas*/*I. setosa*

Plant sources:

Farmers	**Gene bank**	**Researchers**	**Retail stores**
Godollo: No. 1-7	Tápiószele: No. 51-54	Godollo: No. 39-40	Budakeszi: No. 55
Galgahévíz: No. 8-10		Szeged: No. 41-50	Berzence: No. 56-59
Szeged: No. 11-14			
Ásotthalom: No: 15-38			

Plants selected for virus elimination: no. 51-54 from the Hungarian gene bank and no. 25 and 36 from a producer farmer

5.2. **Resistance screening: SPPV-SPCSV co-infection symptoms**

To investigate the resistance mechanism to SPPV-SPCSV complex infection, two sweet potato cultivars, Melinda (ME) and Tio Joe (TJ), were selected out of the 20 screened (Table 1). Symptoms and virus titres were used to screen for resistance and susceptibility to SPPV-SPCSV co-infection in the PT sweet potatoes obtained from Kenya and Hungary. After two independent evaluations, ME was considered susceptible and TJ resistant to SPPV-SPCSV co-infection.

I: Only three out of the twenty inoculated sweet potato cultivars expressed virus symptoms from the fifth wpi (Figure 5) during the first test. ME had vein clearing and downward curling of the top fully open young leaf. Mugande and *Ylw* had severe virus symptoms: stunting, leaf distortion, rugosity, vein clearing, chlorosis and adaxial swelling on their top young leaves after grafting to SPPV-SPCSV infected plant labelled *A6.1*.

(a) Melinda (b) Mugande (c) *Ylw*

Figure 5. Foliar symptoms on infected sweet potato scions. The plants were propagated in the greenhouse, grafted to SPPV-SPCSV infected *A6.1* rootstock, and transferred to a growth chamber. The symptoms were: vein clearing and downward leaf curl on the top fully open young leaf of ME eight wpi (a); Rugosity, vein clearing, leaf distortion, adaxial swelling and chlorosis on young leaves of Mugande eight wpi (b) and *Ylw* twelve wpi (c).

II: TJ was considered a resistant cultivar based on its lack of symptoms and ability to inhibit SPCSV replication and spread regardless of the continuous virus supply from the rootstock. ME, Mugande and *Ylw* were deemed susceptible because they developed virus symptoms and could hardly limit SPCSV accumulation. Many plants died by three weeks post inoculation. Micropropagated Mugande died less than a week after grafting due to grafting stress and accumulation of SPCSV early in the infection (Figure 12). It was discarded after three attempts. Although *Ylw* was symptomatic, it wasn't selected due to lack of information on its agronomic traits, origin and identity. Therefore, ME and TJ cultivars were selected for further investigation based on their symptoms, virus titres, high resistance to SPVD and other desirable agronomic traits according to the sweet potato catalogue (Figures 1, 6 and 12).

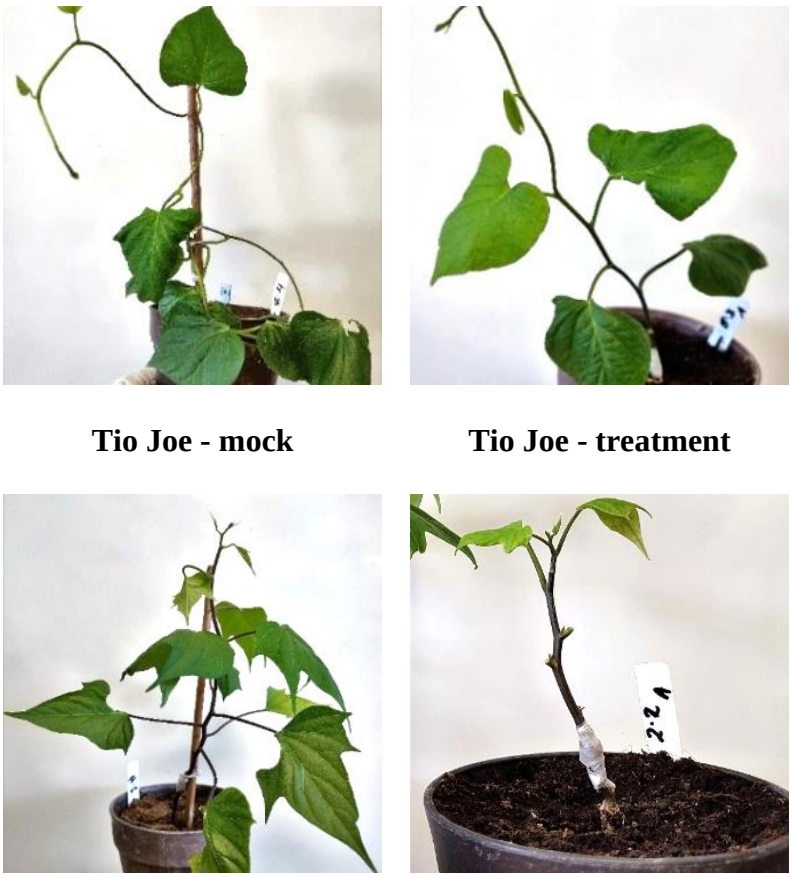


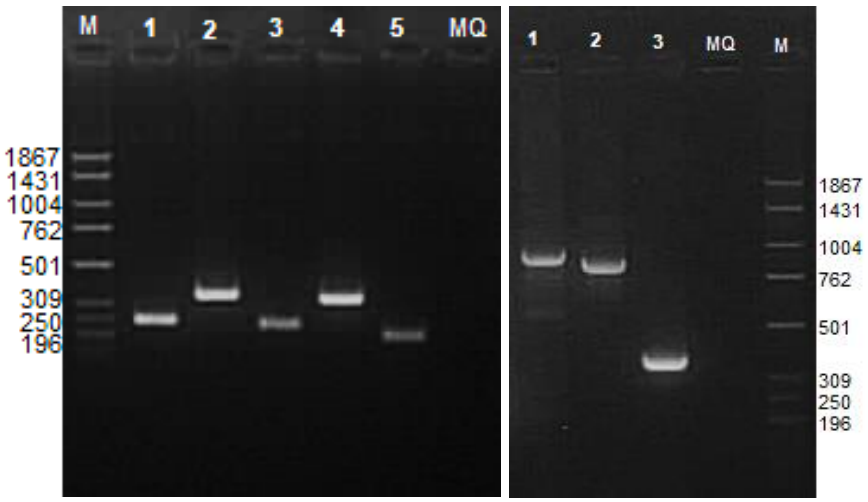
Figure 6. Grafted TJ and ME mocks and their treatments at three wpi. Treatments were grafted to SPPV-SPCSV infected rootstock *A6.1*. The graft-inoculated ME showed mild stunting and chlorosis at three wpi.

5.3. **Transcriptome analysis**

Two biological replicates of TJ and ME treatments and one mock grafted control were selected to investigate the resistance mechanism to SPPV-SPCSV co-infection. An ungrafted TJ was also chosen for comparison with the grafted mock. ME treatments had stunting and leaf chlorosis symptoms at three wpi (Figure 6). There were no phenotypic differences between the grafted and ungrafted TJ controls (data not shown).

5.4. **Molecular virus detection**

Viruses were detected in 54% of tested samples (n=110) from farmers (n=38), researchers (n=12), gene bank (n=4), and retail stores (n=5) (Table 2). Two DNA viruses, namely SPLCV and SPPV, were detected by PCR. Five RNA viruses were detected by qPCR. These were SPCSV – West Africa strain, SPFMV, SPV2, SPVC and SPVG. Eight samples out of the 110 tested positive for SPCSV, five from a farmer's field and three from a retail store (Table 2, Figures 7-9).



(A) (B)

Figure 7. DNA bands amplified by qPCR (A) and PCR (B). A: 1 - sweet potato feathery mottle virus (243 bp), 2 - sweet potato virus C (350 bp), 3 - sweet potato virus G (229 bp), 4 - sweet potato virus 2 (331 bp), 5 - sweet potato chlorotic stunt virus (194 bp). B: 1 - sweet potato leaf curl virus (935 bp), 2 - sweet potato pakakuy virus (867 bp), 3 - malate dehydrogenase gene (400 bp, standard control). MQ - negative control, M - DNA marker.

Up to 66% of the infections involved two or more virus complexes. Only two plants were infected with six viruses, whereas 20 had single viral infections (Table 2). Plants collected from farmers' fields contained all seven viruses detected in different combinations (Table 2, Figure 8), including SPFMV and SPCSV complex, responsible for SPVD. The most severe symptoms were caused by infections of SPCSV complexes in various plants (Table 2).

Six of these viruses occurred in all seven locations where samples were collected except for SPCSV, which was found only in samples from a retail store in Berzence and a farmer's field in Ásotthalom. SPPV and SPFMV were the most abundant viruses detected in 69% and 49% of the infected samples, respectively (Table 2, Figure 8).

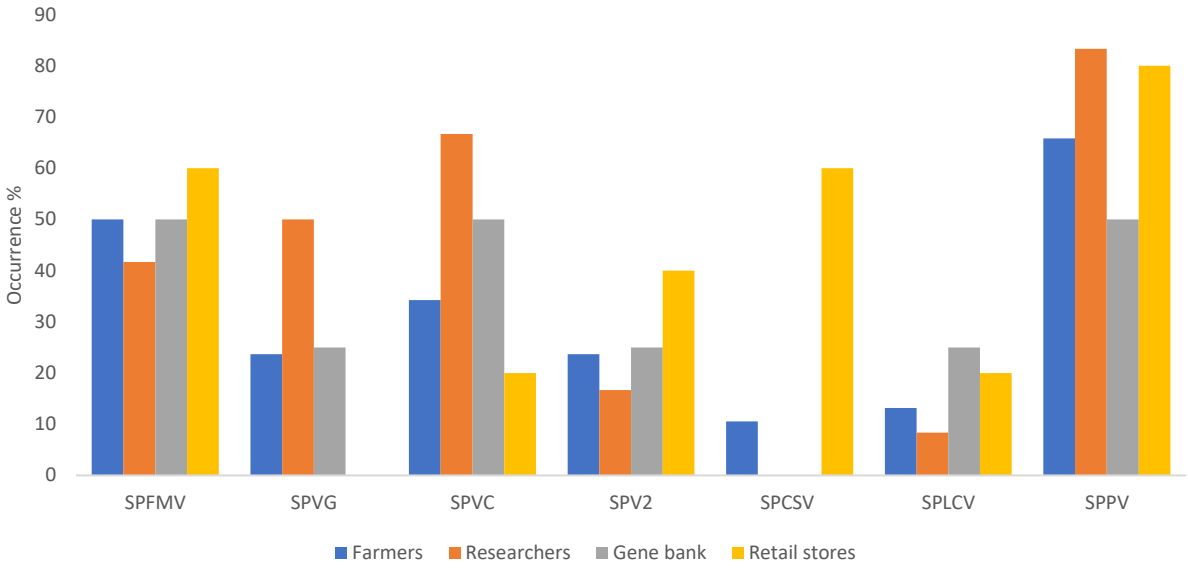


Figure 8. Virus prevalence in sweet potatoes from different sources. Sweet potato feathery mottle virus (SPFMV), sweet potato virus C (SPVC), sweet potato virus 2 (SPV2), sweet potato leaf curl virus (SPLCV), and sweet potato pakakuy virus (SPPV) were present in all four locations where samples were collected.

5.5. **Bioassay**

I. setosa grafted with virus infected sweet potato scions expressed typical viral symptoms from the seventh day after grafting (Table 2). There were no visible symptoms on either the negative control *l. setosa* or the ones grafted with sweet potato taken through virus elimination and tested by PCR and qPCR (Figure 11). Severe stunting and leaf yellowing symptoms developed in *I. setosa* indicator plants grafted with SPCSV infected sweet potato scions. qPCR test for the virus confirmed its presence in the *I. setosa* leaves. More viruses were detected by PCR and qPCR tests in the grafted *I. setosa* than in the donor sweet potatoes. Conversely, SPPV could only be detected in 14% of *I. setosa* grafted with scions containing the virus (Table 2, Figure 9).

Figure 9. Detection of viruses in symptomatic and symptomless sweet potatoes and grafted *I. setosa* indicator plants. Sweet potato chlorotic stunt virus (SPCSV) was the least common, followed by sweet potato leaf curl virus (SPLCV). Transmission of SPPV to *I. setosa* was constrained. Some viruses were detected only after grafting to the indicator plant.

5.6. **SPCSV Hun_01 isolate complete genome**

SPCSV complete genome amplified from sample *A6.1* was sequenced, and the sequences deposited in the GenBank as SPCSV Hun_01 isolate with accessions MW892835 for RNA1 and MW892836 for RNA2. BLASTn search revealed that RNA1 of the Hun_01 isolate had 99.63% sequence identity to SPCSV isolate su-17-10 (acc. MK802073), while RNA2 was 99.68% similar to SPCSV isolate min-17-1 (acc. MK802078) and isolate 24-1 (acc. MK802080). SPCSV Hun_01 isolate had up to 25% sequence difference with the reference sequences acc. NC_004123.1 (RNA1) and NC_004124.1 (RNA2), which are East African strains. Amino acid search showed the presence of SPCSV protein domains in both RNAs. RNA1 contains viral methyltransferase RNA helicase, RNA-dependent RNA polymerase, RNase3 endoribonuclease and p7 domains. RNA2 contains viral Hsp70, Hsp90 homolog, p5.2, p5, p8, p28 and major and minor coat proteins. Phylogenetic analysis using MegAlign (v. 7.1.0, 44) showed a close relationship between Hun_01 isolate and those isolated in China, suggesting they may have a common origin (Figure 10).

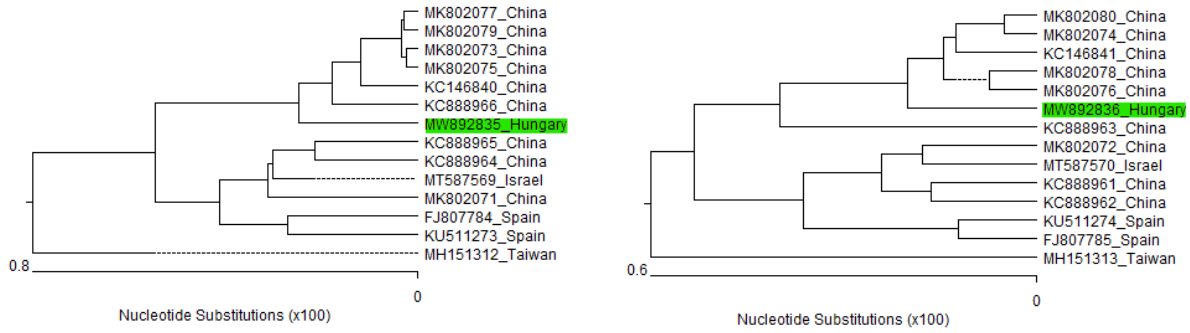


Figure 10. Phylogenetic trees constructed by neighbour-joining method and bootstrapping of 1000 replicates using MegAlign (v. 7.1.0, 44) show evolutionary relationships between SPCSV Hun_01 isolate (highlighted in green) and other closely related isolates from different countries.

5.7. **Partial genome sequences**

Besides sequencing of the SPCSV complete genome, amplified fragments of all the viruses detected were cloned and sequenced, and the sequences were submitted to the GenBank (Table 3).

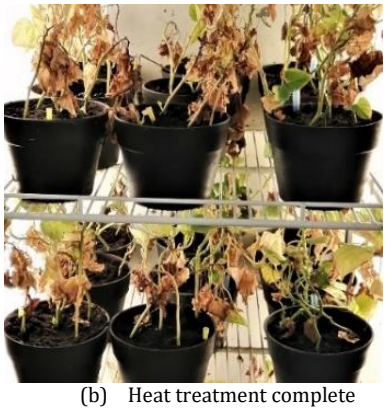
Table 3: Viruses detected, cloned and sequenced

Virus	GenBank Accession	Sample ID
SPCSV	MZ931283	USBer 1
	MZ931284	USBer 2
	MZ931285	USBer 3
SPFMV	MZ931280	T96
SPV2	MZ962674	Gz2.2
SPVC	MZ931281	A10
SPVG	MZ931282	92R
SPLCV	OK094713	A10
	OK094714	USBer 3
SPPV	MZ931279	92R

5.8. **Virus elimination**

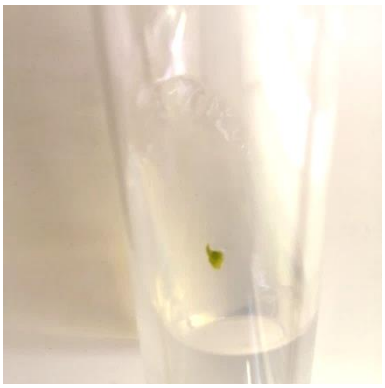
The plants selected for virus elimination were infected by at least two of these viruses: SPFMV, SPVG, SPVC, SPV2, SPLCV and SPPV (Table 2, 4). Only 50% of the plants (n=180) survived the heat treatment and meristem tip culture. The six cultivars, *T96, 92R, 105R, 12R, Blk* and *Ylw*, formed shoots and roots in half-strength MS media at different rates (Figure 11).

(a) Beginning of heat treatment



(b) Heat treatment complete

(c) Sterilized shoot ready for meristem tip cutting



(d) Meristem tip in half-strength MS media

(e) Callus forming shoots

(f) Roots forming

(g) Healthy *I. setosa*

(h) *Ylw* heat treated - 3 weeks after grafting to *I. setosa*

(i) *T96* heat treated - 3 weeks after grafting to *I. setosa*

Figure 11. Different stages of heat therapy and meristem tip culture (a-f); Healthy symptomless *I. setosa* leaf used a negative control (g); Symptomless *I. setosa* grafted with heat treated *Ylw* (h) and *T96* (i) cultivars, which had been tested by PCR and qPCR. *Ylw* was virus free, while *T96* contained SPPV, which was not SPPV transmitted to the indicator plant.

Fifteen plantlets of each cultivar were acclimatized from the media, grown in the greenhouse, and tested for the presence of viruses by PCR and qPCR. The tests showed that only 13% of the plants (n=86) from *T96, 92R, 12R, Blk,* and *Ylw* cultivars turned out free of all viruses except SPPV (Table 4). SPPV was never eliminated from the plants that contained it. These 11 plants were grafted to *I. setosa,* but no symptoms developed within eight weeks.

Leaves from the *I. setosa* were tested for viruses by PCR and qPCR to confirm the absence of viruses. No virus was detected in the symptomless *I. setosa* grafted with scions from sweet potatoes taken through virus elimination. SPVG and SPVC persisted in sample *105R* after the heat treatment. Only SPLCV was not detected in the heat-treated plants; the other viruses could not be eliminated in some plants across the cultivars.

Table 1. Viruses detected in plants before heat treatment and their elimination rate

Plant source	Sample ID	Viruses detected before heat treatment						Virus elimination %
		SPFMV	SPVG	SPVC	SPV2	SPLCV	SPPV	
Gene bank	*105R*	+	+*	+*	+			0
	92R	+	+	+	+		+*	7
	T96	+	+	+	+		+*	13
	12R					+	+*	7
Producer farmer	*Ylw*	+		+			+*	27
	Blk	+		+	+		+*	13

Note: + virus present

+*: Viruses not removed from the cultivars

5.9. **Resistance screening: SPPV and SPCSV titres**

I: Virus disease symptoms development was linked to high SPPV and SPCSV concentrations, as confirmed at nine wpi in ME and *Ylw* in the first evaluation. SPPV was present in all the samples, although its titre was always less than SPCSV (Figure 12). The highest virus concentration occurred in Mayai and Melinda at nine wpi and Mugande and Ndamirabana at three wpi. Sinia B always had the least virus levels. In TJ, there was a gradual decrease in SPCSV titres from the third to the twelfth wpi when it contained one of the lowest amounts of both viruses among the ten samples.

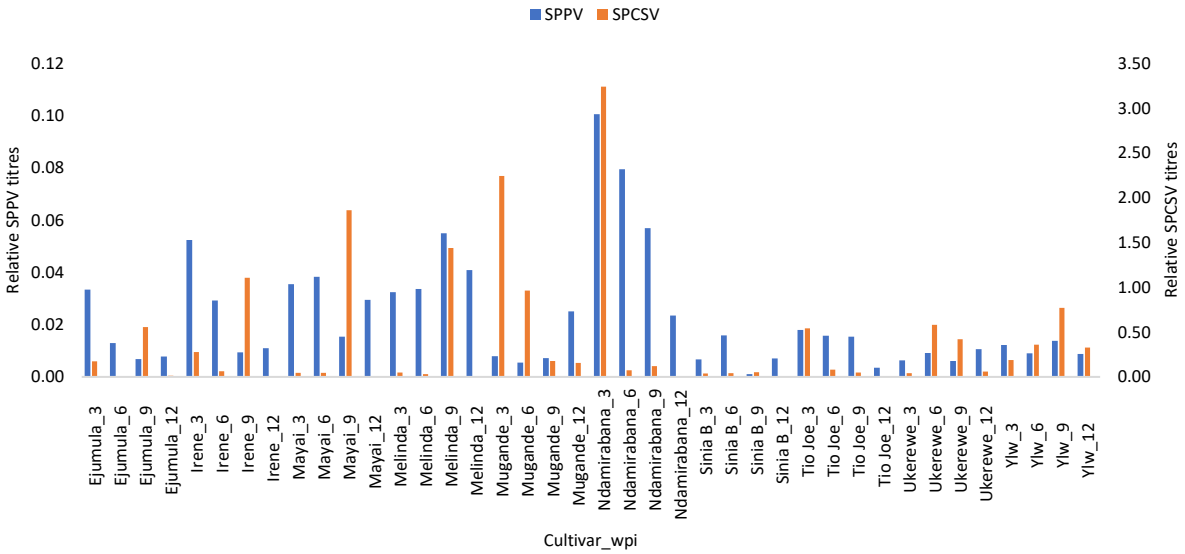


Figure 12. Relative SPPV and SPCSV titres in the top fully open leaves of ten cultivars selected for the second test as measured by one-step qPCR. The tests were done on the 3rd, 6th, 9th and 12th wpi. ME and *Ylw* had their highest SPCSV titres on the ninth wpi. Virus titres in TJ gradually reduced from the 3rd to the 12th wpi. Cq values were normalized to actin.

II: RNA was isolated at one and three wpi in the second test. Virus titres were consistent in tests I and II at three wpi (Figure 12, 13). SPCSV was not detected in any sample by qPCR one wpi. At three wpi, there was more SPCSV in treated ME_3 than in TJ_3. SPPV titres were very low compared to SPCSV. SPPV expression was up to 6.5 folds lower than actin. There was a positive correlation between SPPV and SPCSV concentrations in both cultivars. As in the first test, ME contained more SPPV and SPCSV than TJ. The treatments had more SPPV than their mocks (Figure 13). The difference in SPPV content of grafted and ungrafted TJ at one wpi was negligible (data not shown).

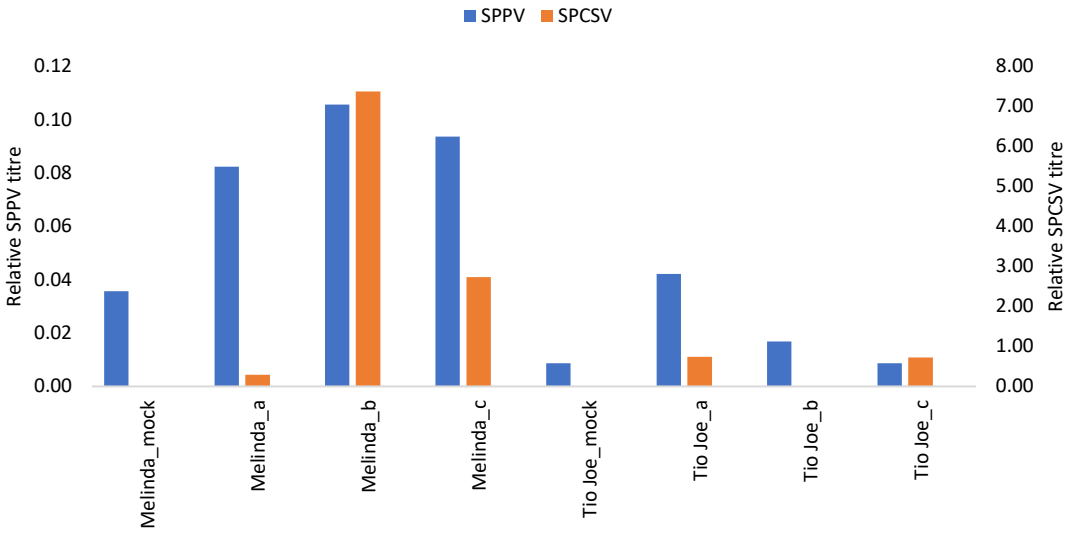
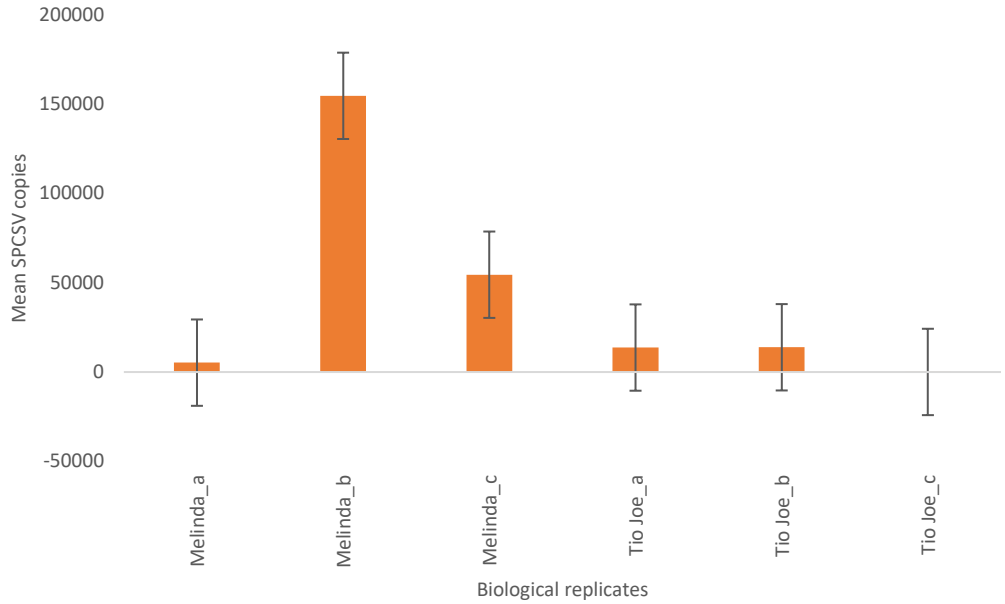


Figure 13. Concentration of SPPV and SPCSV in mocks and three biological replicates (a,b,c) of ME and TJ treatments at three wpi. ME_3 had higher virus titres compared to TJ_3. A positive correlation coefficient of 0.7 was recorded between SPPV and SPCSV average relative titres, indicating possible synergy.

BLASTn search confirmed the PCR amplification of SPPV sequences in all the samples. The rootstock *A6.1* contained SPCSV Hun_01 isolate.

SPCSV copy numbers were calibrated from a cloned plasmid containing a known concentration of SPCSV using a standard curve. All the mocks lacked SPCSV. The variation in SPCSV quantities in the three biological replicates signifies differences in plant-pathogen interactions between individual plants of the same cultivar. ME_3 had an average of eight times more SPCSV than TJ_3. A lower SPCSV amount could be associated with resistance in TJ_3 (Figure 14).



Error bars: standard error

Figure 14. SPCSV virus copies in the three biological replicates of ME_3 and TJ_3 fully open top young leaves. Unlike in TJ_3, there was a considerable variation in the virus quantity between ME_3 biological replicates, signifying individual differences in plant-pathogen interactions within a cultivar. On average, ME_3 contained eight times more SPCSV than TJ_3, suggesting susceptibility.

5.10. **RNAi genes expression analysis**

SPPV pre-infection in the source PT plants was suspected of inducing defence genes expression and affecting graft transmission of its cognate virus through siRNA cross-protection. Expression analysis of five RNAi genes, namely DCL2, DCL4, AGO1, AGO4 and SDE5, showed similar patterns in TJ and ME one and three wpi (Figure 15). Gene expression was evaluated in three biological replicates of ME and TJ treatments and their controls with two technical replicates using the delta-delta Cq method and actin as the reference gene.

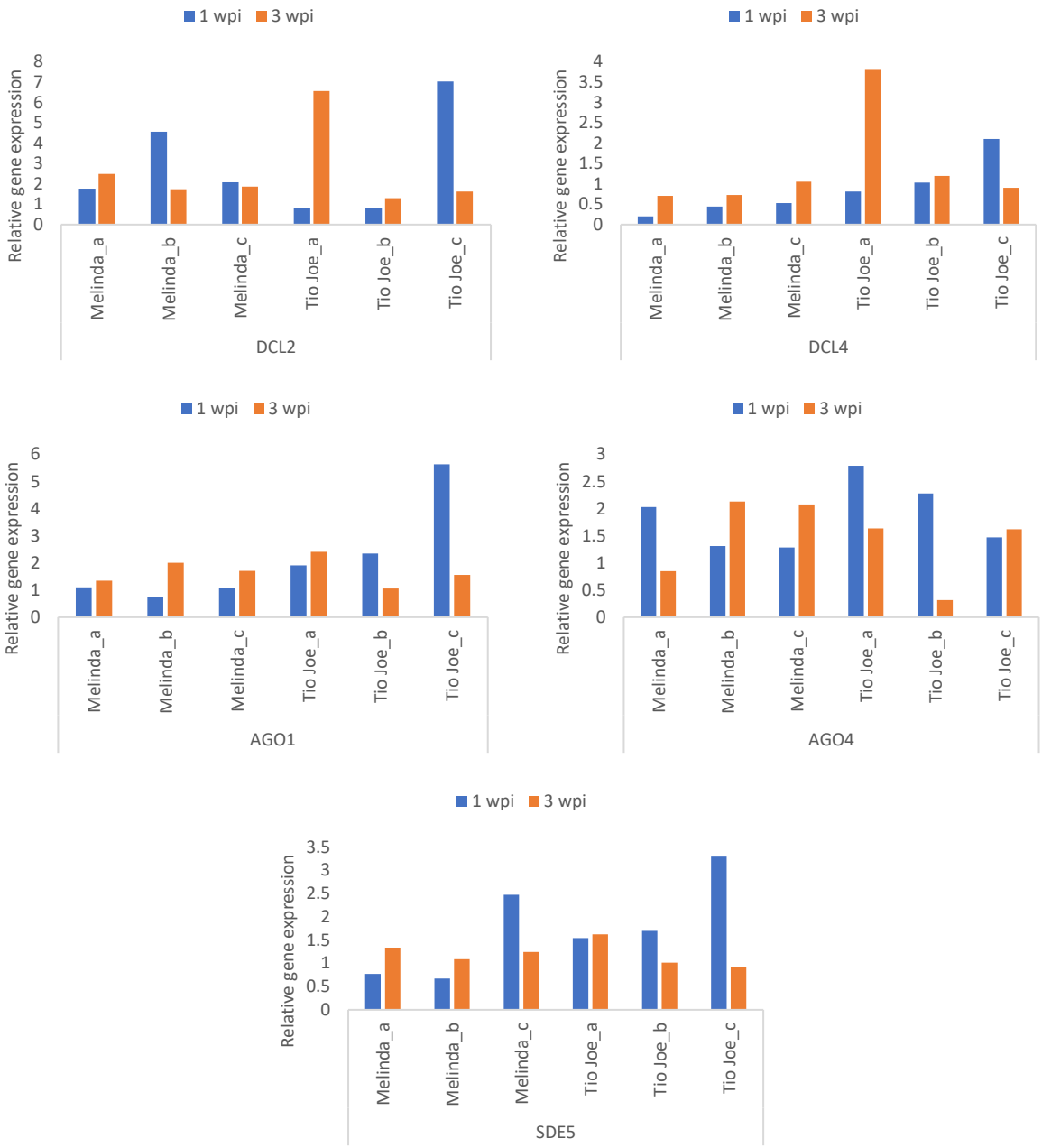


Figure 15. Expression of RNAi genes DCL2, DCL4, AGO1, AGO4 and SDE5 in three biological treatments (a, b, c) relative to their mocks. The relative gene expression levels were not high, and their patterns in ME and TJ were similar.

5.11. **Transcriptome analysis: Reads mapping to the reference genome**

After reads trimming, the four treatments and their mocks had a mean of 201 million reads with an average length of 131 nucleotides. The average number of trimmed reads mapped to the reference genome was 152 million (75.53%), of which 88.85% were paired reads with a mean distance of 148.53. Paired reads that mapped to the *I. trifida* genome were 55.3% on average. Fragments that mapped to genes in the reference genome and were used for gene expression analysis were 61%. Broken pairs, which were ignored were on average 7.9% of the reads. De novo

assembly produced 30,619 contigs with an average length of 356 nucleotides per sample. An average of 98.41% trimmed reads mapped to the contigs, of which 79.85% were paired (Table 5).

Table 5. High throughput sequencing data quality

Sample ID	Read count (mapped)	Avg. length (after trim)	Mapped reads	Mapped pairs (%)	Mean distance
Mock ME_1	179 791 496	130.32	134 478 543	54,88	146.38
Mock_TJ_1	237 952 520	129.22	178 365 553	56,00	144.49
Mock_ME_3	177 618 064	131.63	135 385 596	55,47	149.68
Mock_TJ_3	197 533 830	132.03	147 285 325	54,51	149.98
Trt_ME_1	202 984 332	130.38	154 188 746	56,07	146.90
Trt_ME_3	229 161 438	132.12	176 864 697	55,68	150.95
Trt_TJ_1	199 015 572	132.16	144 561 425	53,67	149.63
Trt_TJ_3	188 967 312	131.82	147 213 413	56,34	150.20

5.12. **Virus check in the transcriptome**

Reference sequences used to search for viruses with the best BLASTn hits were: sweet potato pakakuy virus acc. NC_015655, sweet potato chlorotic stunt virus RNA 1, acc. NC_004123.1, sweet potato chlorotic stunt virus RNA 2, acc. NC_004124.1, and sweet potato symptomless mastrevirus 1 (SPSMV-1) acc. NC_034630.1. BLASTn search of the consensus virus sequences showed that:

a) SPCSV in ME_3 and TJ_3 was up to 99.98% similar to the Hun_01 isolate contained in sample *A6.1*, which was the inoculum source.
b) All the samples contained sweet potato badnavirus B, which was 96% similar to Chinese isolate acc. MK052980.1.
c) Suspected SPSMV-1 sequences were similar to *I. trifida* genome; hence it was a false positive. PCR and qPCR ruled out the presence of SPSMV-1 in all the samples.

Both mocks and treatments contained SPPV. SPPV reads reduced slightly in all mocks and TJ three wpi but increased in ME_3. There were very few SPCSV reads in the treatments at one wpi, which increased considerably at three wpi. At three wpi, ME_3 had more SPCSV reads than TJ_3, and SPPV reads were much lower than SPCSV, consistent with the qPCR results. The reads fully covered the SPCSV genome, whereas SPPV wasn't (Figure 12-14, Appendix 12.3).

5.13. **Differential gene expression**

There was a big difference between the mocks and treatments in both cultivars, more so in ME, one wpi, which could be attributed to SPPV_SPCSV infection. At three wpi, there was less variation between mocks and treatments (Figure 16).

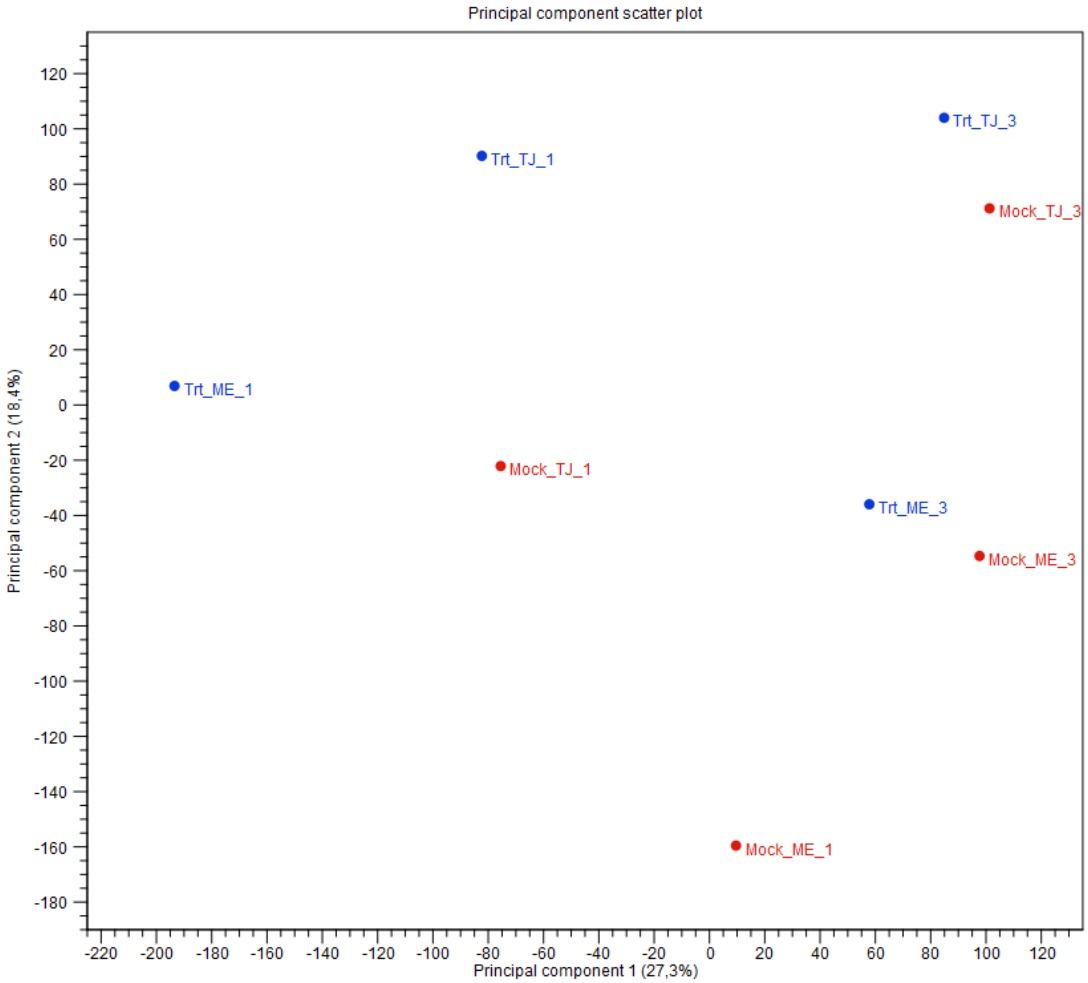


Figure 16. The huge difference between mocks and treatments one wpi could be attributed to the virus infection. Reduced variation between mocks and treatments three wpi could be associated with virus tolerance.

The biggest overlap of DEGs occurred between TJ_1 and ME_1. Differential gene expression was highest in ME_1, followed by TJ_1. ME_1 had the most DEGs at 8105, while TJ_3 had the least at 3240. Common overlapping DEGs between ME_1 and TJ_1 were 3252; between ME_3 and TJ_3, there were 983 DEGs (Figure 17, 18).

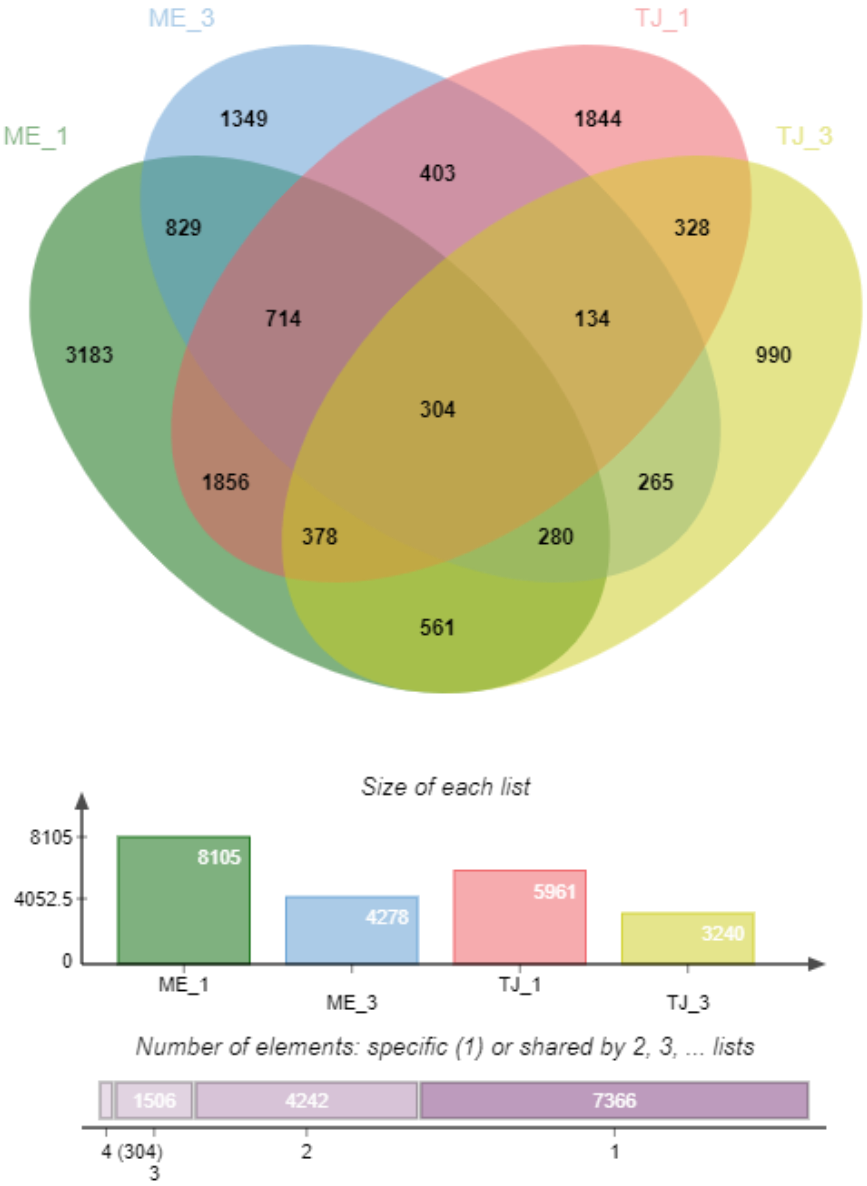
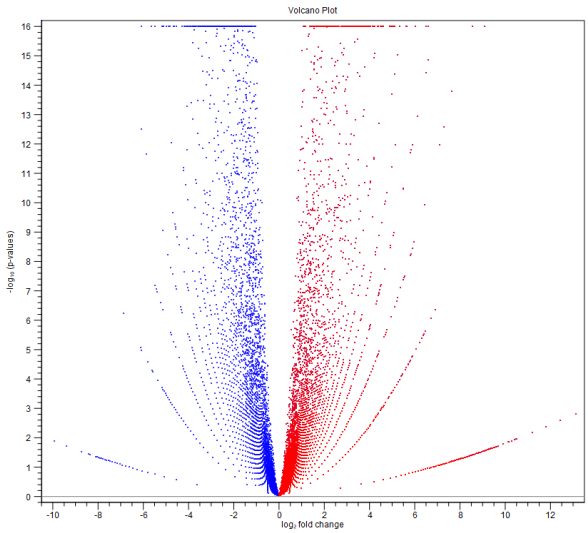
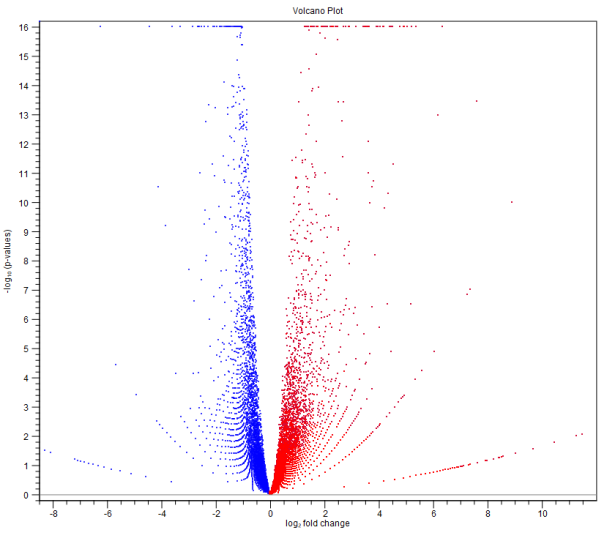


Figure 17. Venn diagram showing overlap of DEGs between ME and TJ one and three wpi. The biggest overlap was in ME_1 and TJ_1. Values represent the number of DEGs between mock and treatment with a fold change ≥ 1.5 and p-value ≤ 0.05.

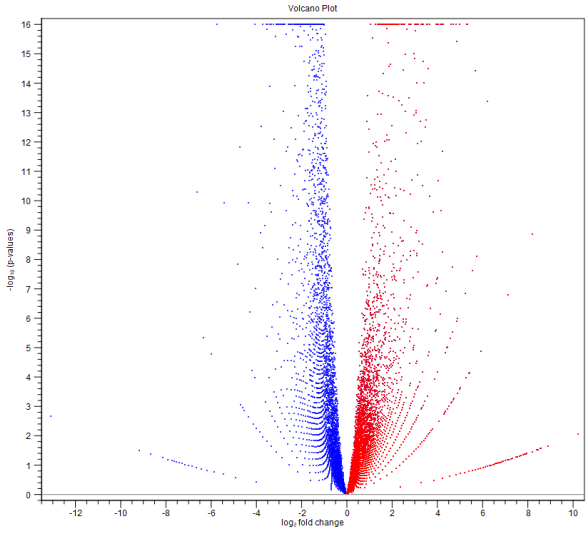
At one wpi, most DEGs were downregulated. The trend was reversed at three wpi when more genes were upregulated to overcome the infection in both cultivars (Figure 18).



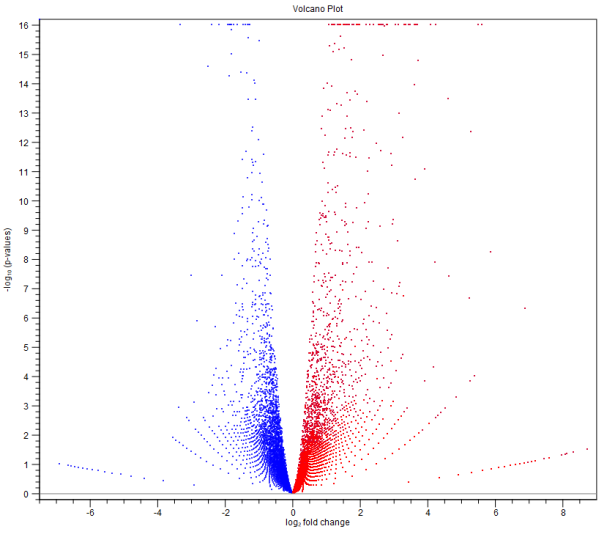
ME_1 (DEGs 8105, Up 3837, Down 4268)



ME_3 (DEGs 4278, Up 2146, Down 2132)



TJ_1 (DEGs 5961, Up 2658, Down 3303)



TJ_3 (DEGs 3240, Up 1829, Down 1411)

Figure 18. Volcano plots show that differential gene expression was highest in ME_1 and lowest in TJ_3. The total number of DEGs, those regulated up (red) and down (blue) with a fold change ≥ 1.5 and p-value ≤ 0.05, are shown in brackets.

DEGs induced in response to viruses to enhance defence responses against them are often targeted by the viruses to inhibit their transcription and translation. Viruses inhibit defence and growth gene expression in susceptible plants, but resistant ones prevent or overcome such attacks. The defence genes overexpressed in TJ_1, but not ME_1 could account for the early virus resistance in TJ (Figure 19).

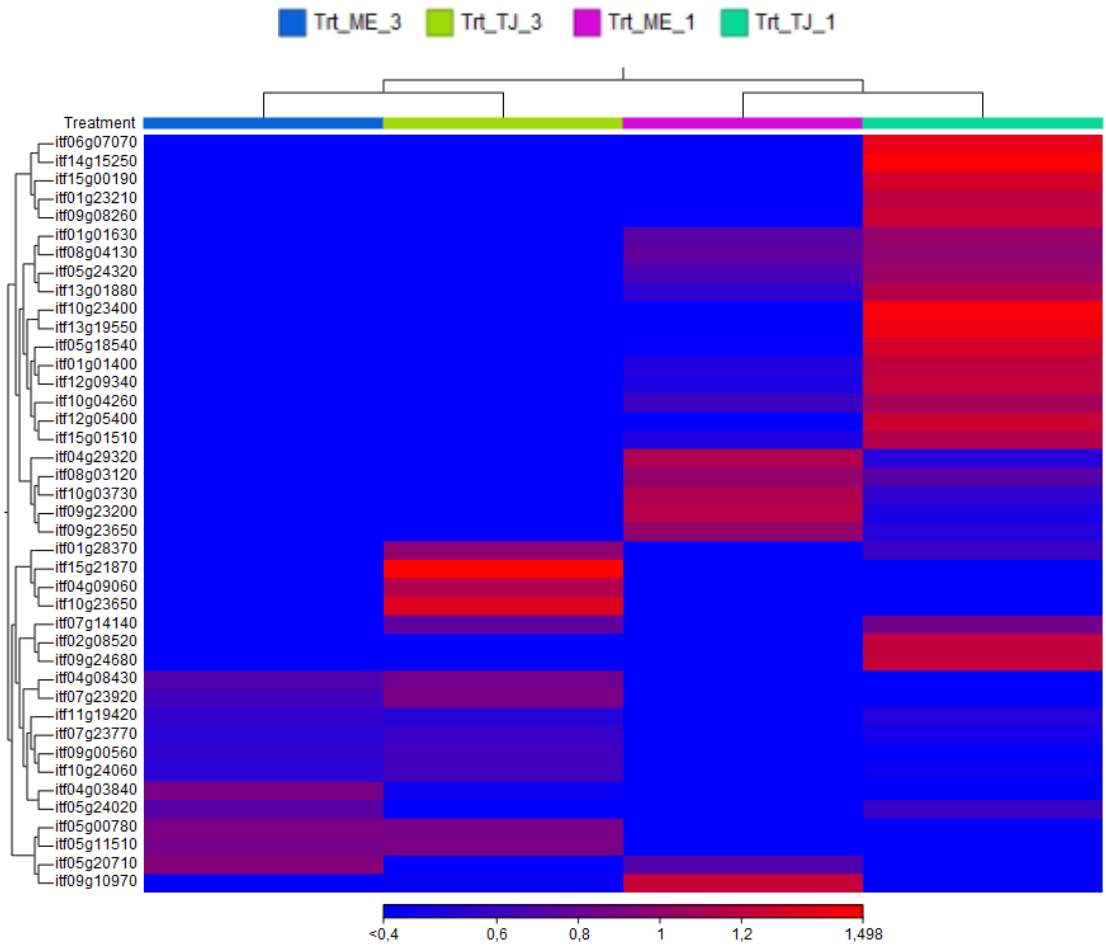


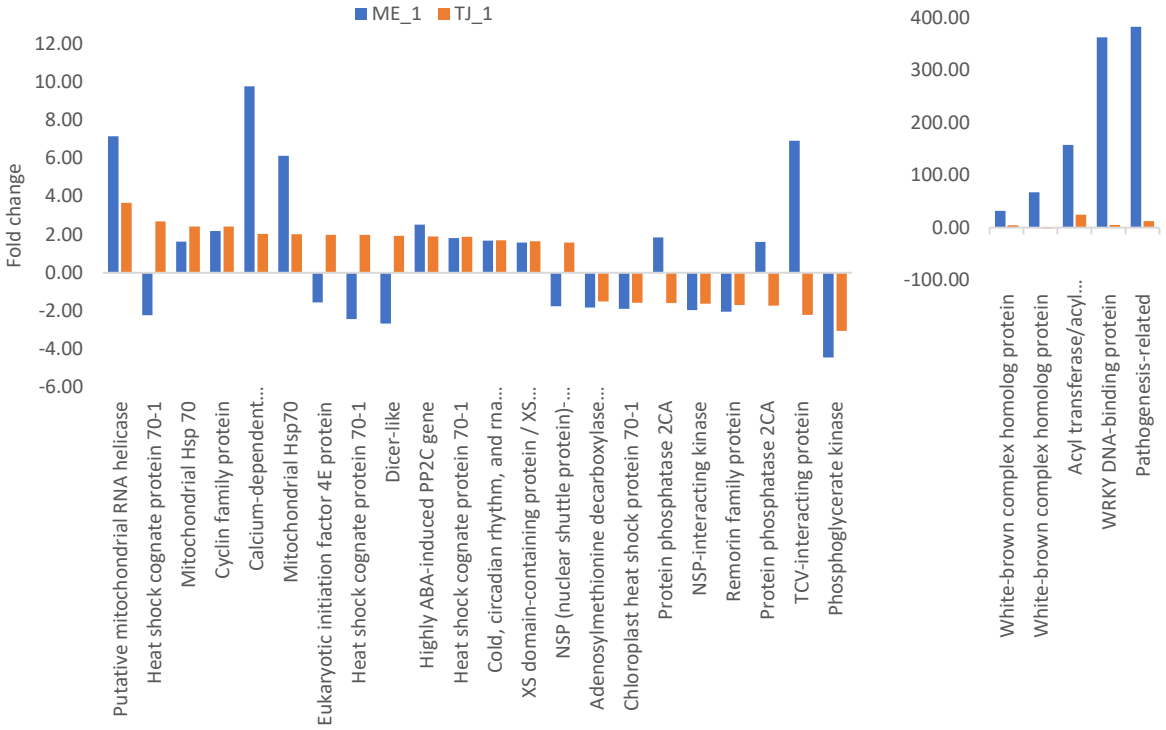
Figure 19. Heat map showing clustering and differential expression of selected genes between treatments of TJ and ME at one and three wpi. The genes were either overexpressed in TJ or induced in response to the SPPV-SPCSV co-infection.

5.13.1. DEGs responsive to virus infection

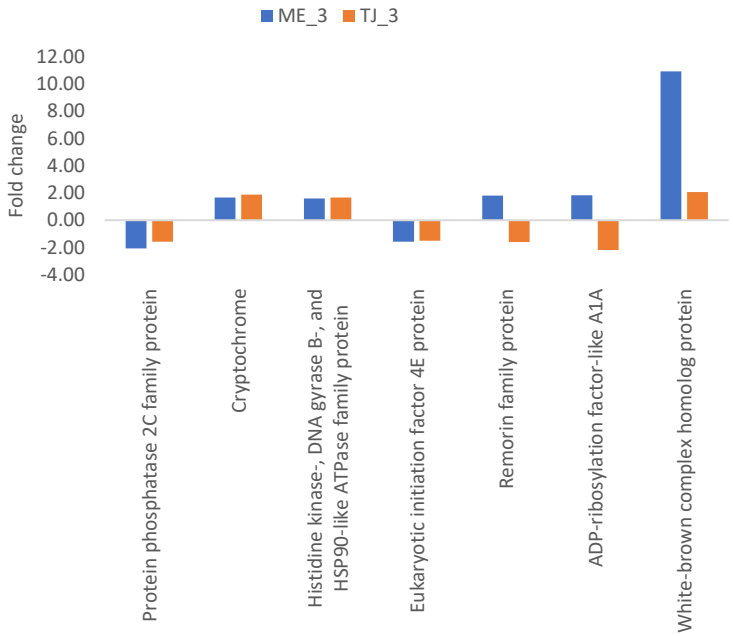
Two RNAi genes were differentially expressed in both cultivars. Dicer-like protein (itf07g23770, DCL4 isoform) was upregulated in TJ_1 and downregulated in ME_1. According to qPCR, DCL4 was overexpressed in TJ than in ME one and three wpi (Figure 15). SGS3 XS domain-containing protein / XS zinc finger domain-containing protein-related (itf05g20710) was upregulated in ME_1 and TJ_1 (Figure 19).

DEGs which respond to viruses were overexpressed in both cultivars one and three wpi. They include heat shock cognate protein 70-1 (itf14g15250, itf09g24680, itf12g09340), mitochondrial Hsp70 (itf10g23400, itf01g01400), cyclin family protein (itf13g19550), eukaryotic initiation factor 4E protein (itf04g08430), cold, circadian rhythm, and RNA binding (itf01g23210), highly ABA-induced PP2C gene (itf04g29320), putative mitochondrial RNA helicase (itf09g08260), acyl transferase/acyl hydrolase/lysophospholipase superfamily protein (itf08g04130), WRKY DNA-

binding protein (itf10g03730), pathogenesis-related gene (itf09g23200) and protein phosphatase 2C (PP2C) family protein (itf01g01630) (Figure 19, 20).



(a)



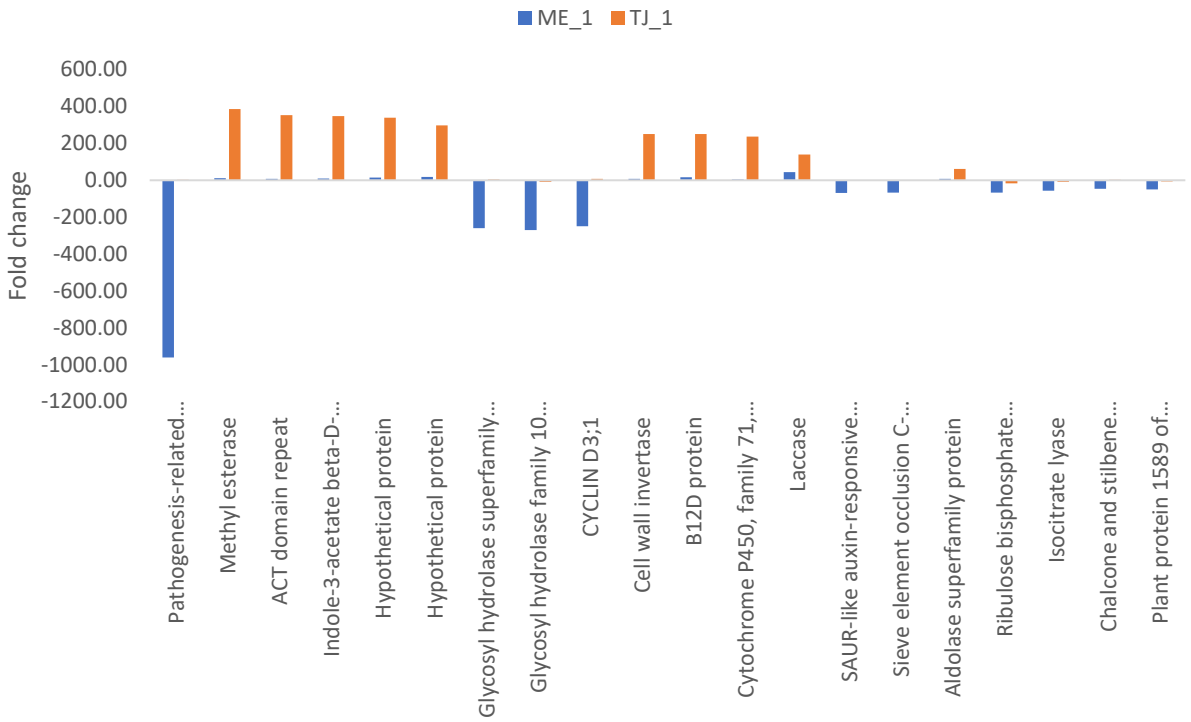
(b)

Figure 20. Differential expression of genes induced in response to viruses one (a) and three (b) wpi. Graph (a) was split for clarity.

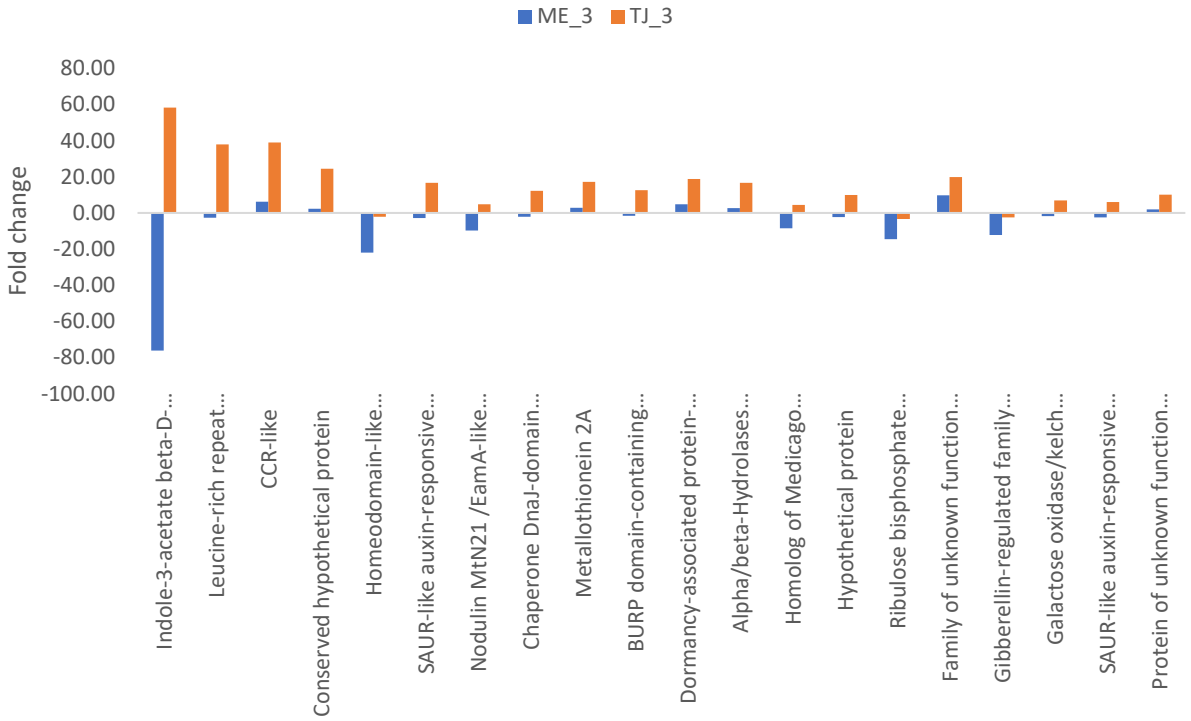
5.13.2. **DEGs overexpressed in Tio Joe**

DEGs overexpressed in TJ_1, some of which could be responsible for SPPV-SPCSV resistance, include pathogenesis-related thaumatin superfamily protein (itf09g00560), ACT domain repeat (itf05g18540), cyclin D3;1 (itf10g24060), chalcone and stilbene synthase family protein (itf04g09060), pectin lyase-like superfamily protein (itf05g24020), plant nuclear matrix protein 1 (NMP1) domain containing protein (itf04g03840), cyclin-dependent kinase B2;2 (itf07g23920), indole-3-acetate beta-d-glucosyltransferase (itf01g28370), methyl esterase (itf05g11510, itf12g05400), cinnamate-4-hydroxylase (itf07g14140), cell wall invertase (itf15g01510), methyltransferases (itf10g23650, itf11g19420) and phenylalanine ammonia-lyase (itf06g07070, itf02g08520, itf15g00190), which is involved in biosynthesis of SA, flavonoids and aromatic amino acids (Figure 21).

DEGs overexpressed in TJ_3 include CCR-like (itf05g00780), SAUR-like auxin-responsive protein family (itf09g23650), nodulin MtN21 /EamA-like transporter family protein (itf09g10970), chaperone DnaJ-domain superfamily protein (itf08g03120), metallothionein 2A (itf07g19700) and BURP domain-containing protein (itf15g21870) (Figure 21).



(a)



(b)

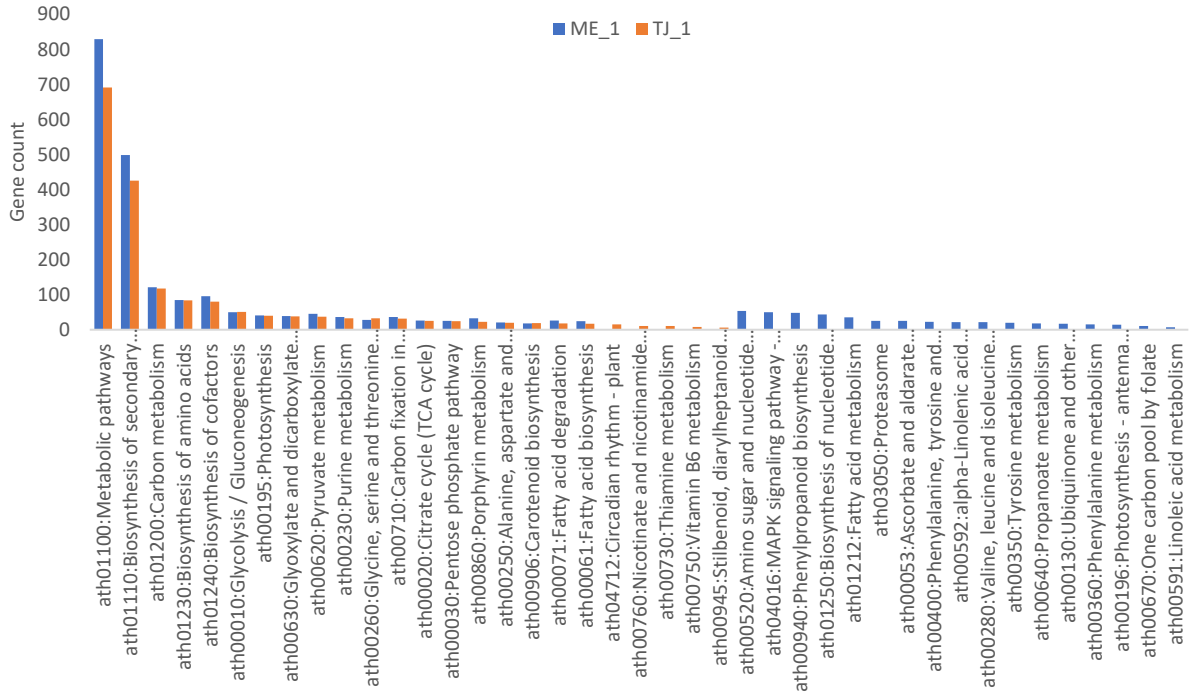
Figure 21. Top 20 DEGs overexpressed in TJ compared to ME at one (a) and three (b) wpi.

5.14. **Gene set enrichment analysis**

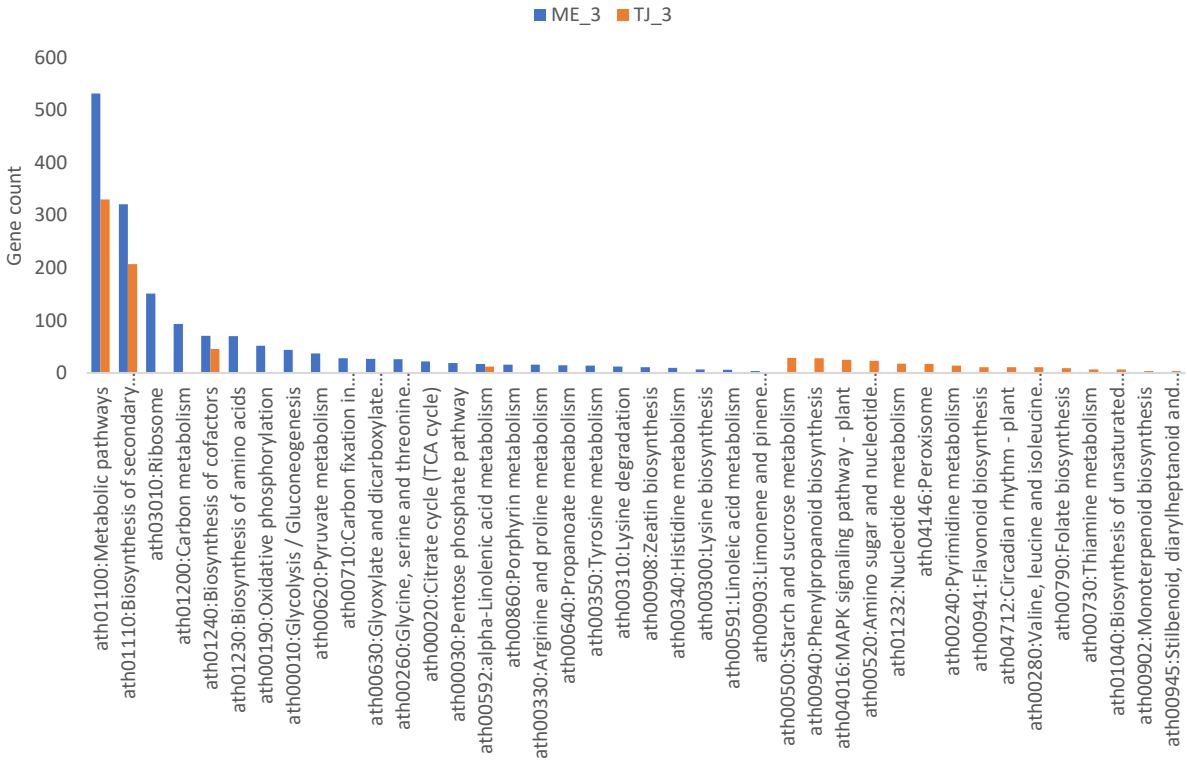
According to the Kyoto encyclopedia of genes and genomes (KEGG), pathways enriched in TJ_1 include metabolism of vitamins nicotinate, nicotinamide, thiamine, and vitamin B6; in addition to biosynthesis of phenolic compounds, stilbenoid, diarylheptanoid and gingerol (Figure 22).

KEGG pathways enriched in TJ_3 were starch and sucrose metabolism, phenylpropanoid biosynthesis, MAPK signalling pathway, amino sugar and nucleotide sugar metabolism, nucleotide metabolism, peroxisome and pyrimidine metabolism pathways (Figure 22).

Biosynthesis of secondary metabolites, cofactors and carbon metabolism were enriched at both intervals in TJ and ME.



(a)

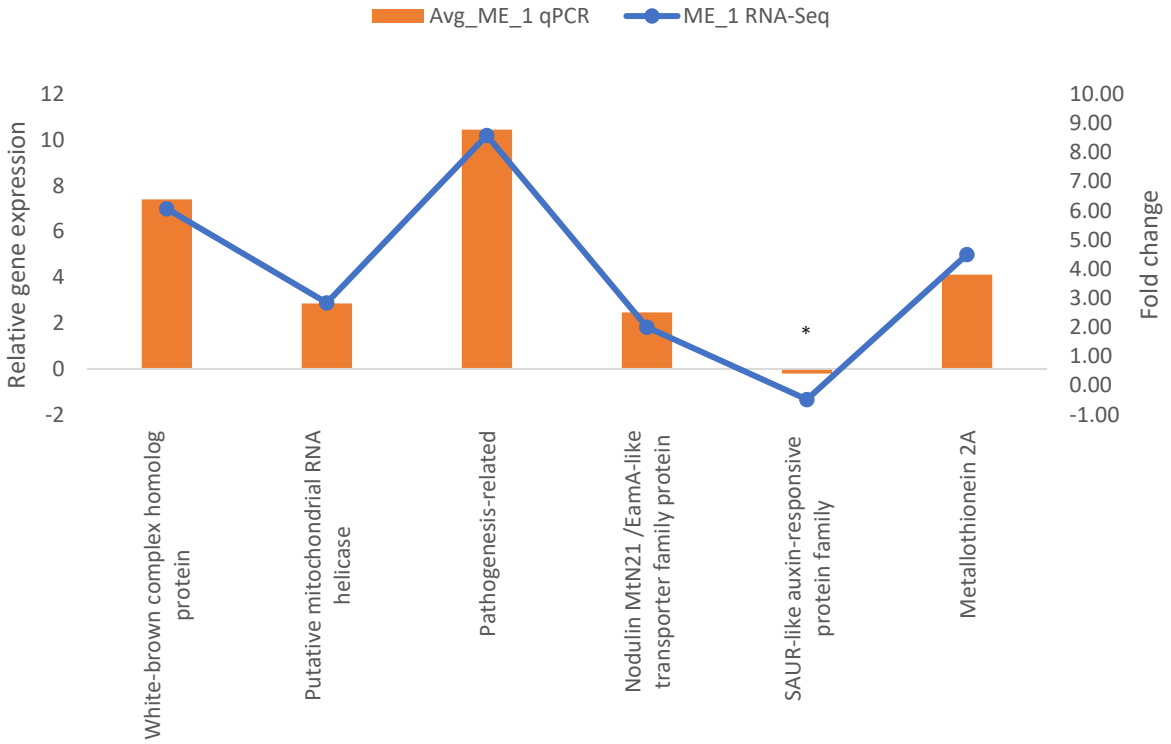


(b)

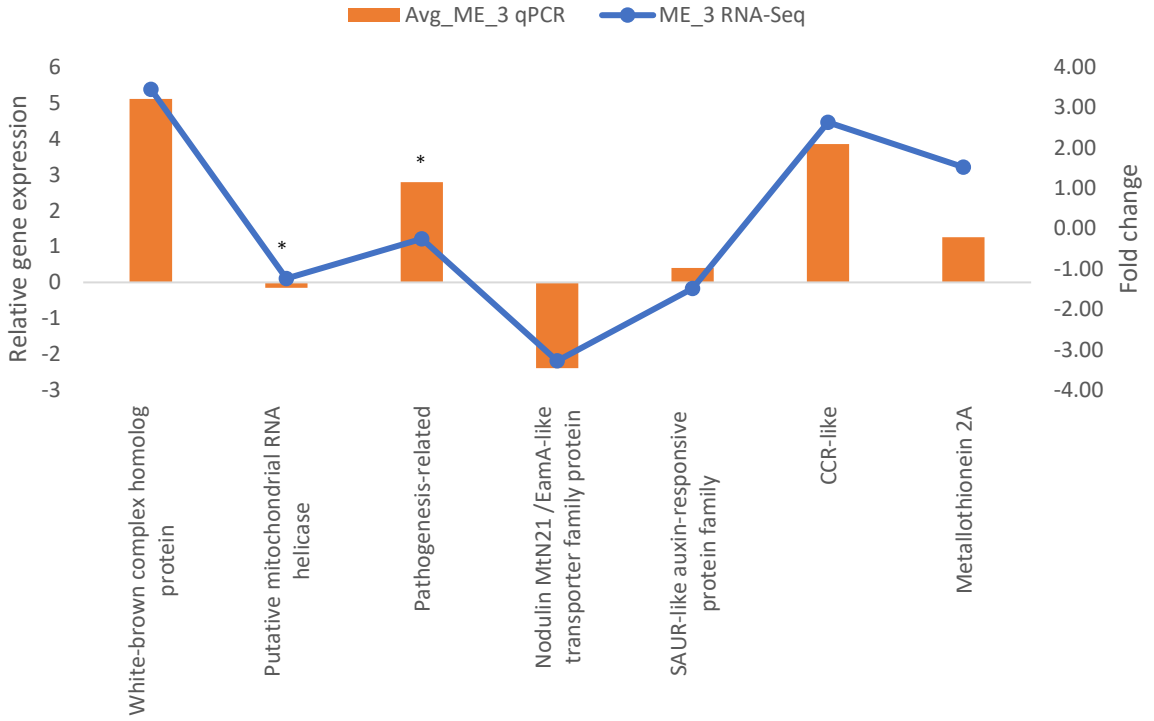
Figure 22. KEGG pathways enriched in TJ and ME one (a) and three (b) wpi. ath - prefix of Arabidopsis KEGG pathway entry number

5.15. **Validation of RNA-Seq by qPCR**

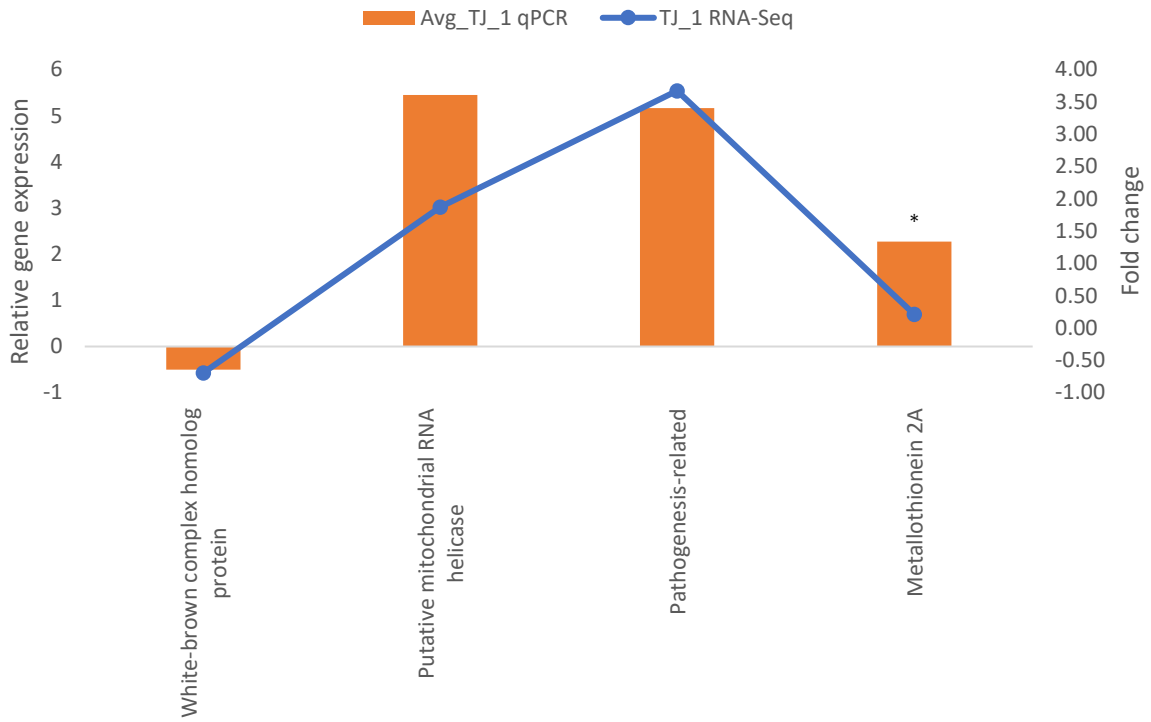
Seven DEGs were randomly selected and amplified by qPCR in the three biological replicates of TJ and ME with two technical replicates. Delta-delta Cq gene analysis of their average expression levels showed the same pattern as in RNA-Seq, confirming the validity of the HTS and transcriptome data analysis (Figure 23).



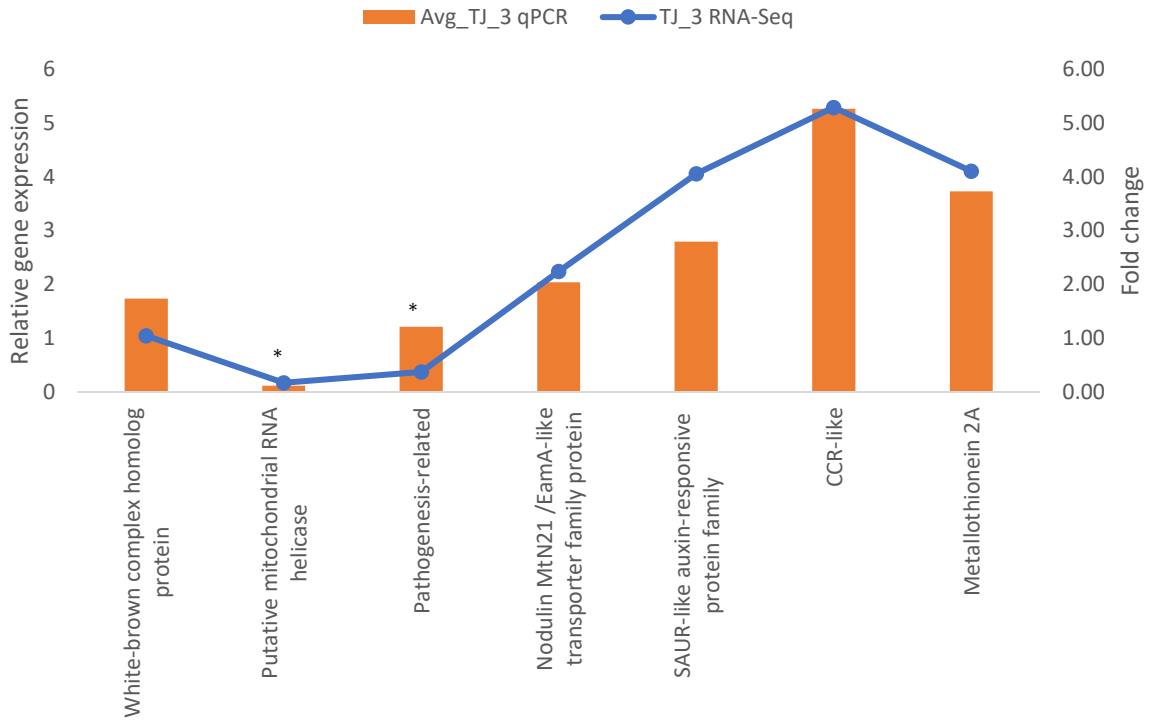
(a)



(b)



(c)



(d)

* p-value > 0.05, insignificant

Figure 23. Comparison of RNA-Seq and qPCR gene expression data in selected DEGs showing similar expression patterns in ME_1 (a), ME_3 (b), TJ_1 (c) and TJ_3 (d), which confirms the validity of the HTS and transcriptome data analysis.

Very low reads of SPCSV one wpi, their increase three wpi and the invariable low titre of SPPV in the first and third wpi were consistent in RNA-Seq and qPCR analysis (Figure 12-14, Appendix 12.3)

6. DISCUSSION

6.1. Virus detection in sweet potatoes in Hungary

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 genera (Appendix 12.2.1). Seven viruses were detected: SPCSV, SPFMV, SPV2, SPVC, SPVG, SPLCV and SPPV. This is the first report on the occurrence of SPCSV (Kiemo et al., 2022a), SPPV, SPLCV and sweet potato virus disease (SPVD) caused by SPFMV and SPCSV synergistic infection in sweet potatoes in Hungary (Kiemo et al., 2022b).

Sweet potato viruses cannot be detected, distinguished, or identified based on visible symptoms because the viruses often occur in mixed infections or cause similar symptoms. Single virus infections seldom cause visible symptoms (Untiveros et al., 2007). Potyviruses cause chlorotic spots, while SPFMV causes feathery mottles in the veins. SPLCV is mostly symptomless but leads to yield reduction. Infection of SPCSV with other RNA and DNA viruses causes the most severe viral disease symptoms in sweet potatoes due to synergism. In these cases, SPCSV titres decrease or remain the same while the other viruses increase significantly. There is no correlation between symptom severity and the number of viruses in a plant. The severity of the symptoms is 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 of potyviruses and badnavirus SPPV could cause the prevalence of these viruses in sweet potatoes as farmers and producers unknowingly select infected plants for propagation (Gibson et al., 1997; Gibson and Kreuze, 2015; Kreuze et al., 2020). This results in virus accumulation in consecutive seasons resulting in cultivar degeneration which is 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 is 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).

6.2. 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 reginal 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 (36^{o}C/16 hours and 32^{o}C/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). Virus elimination programs should (if possible) avoid SPPV infected plants, although this may not be easy because it is ubiquitous in the germplasm (Clark et al., 2012; Kreuze et al., 2020; Kiemo et al., 2022b). It is intriguing how SPPV persists in newly formed meristematic cells during heat treatment. SPLCV, a seed-borne DNA virus like SPPV, is readily transmitted by grafting to *I. setosa* and eliminated from sweet potatoes by heat treatment, unlike SPPV. 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; Kiemo et al., 2022b). 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 (Kiemo et al., 2022b). The transmission efficiency of SPPV variants (sweet potato badnavirus A and sweet potato badnavirus B) to *I. setosa* differs and is also limited by other viruses like SPFMV and SPCSV (Kreuze et al., 2020). It could also be cultivar dependent. Grafting is the

ultimate mode of inoculating viruses that are not easy to transmit mechanically, such as SPCSV and SPFMV. Grafting, however, causes a continuous supply of the pathogen leading to an exaggerated infection pressure which may break the resistance in some cultivars, which were resistant in low virus concentrations (Loebenstein and Carr, 2006).

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

In optimum conditions, the plant defence system will work best to overcome or limit the spread and impact of an infection, especially if the pathogen concentration is low. At temperatures below 15 ^{0}C, viruses will overwhelm most plants because RNAi is almost inactive. RNAi is highest at higher temperatures over 24 ^{0}C; therefore, tolerant or moderately resistant plants can overcome the viral infection (Szittya et al., 2003). Consequently, our plants were maintained at an optimum temperature of 23 ^{0}C to balance virus accumulation and defence responses. Grafting with an infected rootstock provides continuous virus supply to the scion. This can be overcome when resistant scions transfer vsiRNA to the rootstock to silence the virus through RNAi (Loebenstein and Carr, 2006).

An array of biotic and abiotic factors determines symptom development. These include pathogenicity of the virus(es), the plant's physiological condition, growth stage, and the environment it's growing in (Bradamante et al., 2021). The vein clearing, chlorosis, and stunting symptoms expressed in Mugande and *Ylw* cultivars were similar to SPVD (Gibson et al., 1998; Untiveros et al., 2007). Optimum growth conditions *in vitro* and in growth chambers enhanced the plants' fitness, increasing their tolerance to the viruses (Szittya et al., 2003). Ungrafted *A6.1* plants propagated *in vitro* and then in the growth chamber were more vibrant, with fewer virus symptoms than the greenhouse ones (data not shown).

Five weeks after inoculating plants from the greenhouse, ME, Mugande, and *Ylw* cultivars developed disease symptoms which progressed in severity with time. Due to very low titres, SPCSV could not be detected in the first wpi by qPCR. Virus tests by qPCR showed SPPV and SPCSV viruses were present in all the treatments at different concentrations three wpi (Kokkinos and Clark, 2006b). Symptoms gradually developed in ME, Mugande and *Ylw* from the fifth wpi correlating with the high virus titres recorded at six and nine wpi in the first test; hence these cultivars were considered susceptible to the SPPV-SPCSV co-infection (Tavantzis, 1984; Untiveros et al., 2007). The higher virus titres in ME than in TJ were also recorded in the second test. 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).

The amount of SPPV was not different in the TJ and ME. Presence of SPPV in all PT plants was consistent with reports that SPPV is ubiquitous in sweet potato germplasm. Our source plant

materials were pathogen tested in CIP, Nairobi. SPPV poses a phytosanitary risk in sweet potato germplasm transfer across territories. The virus is widespread, possibly because of its persistence in the meristems and recalcitrance to heat therapy (Varveri et al., 2015; Kiemo et al., 2022b). Reduced viral load and virulence have been linked to vertical transmission of viruses whereby trade-offs are made between the plant and the virus to co-exist mutually (Tavantzis, 1984; Paudel and Sanfaçon, 2018; Bradamante et al., 2021). Perhaps this is the basis of symbiosis between SPPV and sweet potato (Roossinck, 2008, 2011, 2012; Bradamante et al., 2021). The trade-offs allow the virus to be transmitted through the seeds at a low fitness cost by the plant, allowing for continued fecundity and survival.

Different SPPV variants are not mutually exclusive, but their transmission through the graft junction can be limited by other viruses, such as SPCSV (Kreuze et al., 2020). Most likely, SPPV was not transferred from the *A6.1* rootstock to the PT scions due to siRNA induced cross-protection since the scion was already infected with the virus (Loebenstein and Carr, 2006; Roossinck, 2010; Bradamante et al., 2021).

Viruses often hijack plant translation machinery to multiply and spread in the host cells to reach detectable levels. Our first test showed that in TJ, SPCSV concentration reduced gradually from the third to the twelfth wpi, pointing to a resistance mechanism hindering the virus replication and spreading, especially to the top young leaves (Tavantzis, 1984; Paudel and Sanfaçon, 2018). In ME, however, SPCSV concentration wasn't constant, as it spiked in the ninth wpi and reduced again in the 12 wpi. Virus overexpression re-ignites defence responses, reducing systemic spread (Jones and Dangl, 2006).

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

6.4. **Virus effects on gene expression**

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.

When pathogens attack, they interfere with many cellular processes in the plant. Defence responses come at a considerable fitness cost for the plant, limiting its growth and development and leading to symptoms such as stunting and low yields (Pumplin and Voinnet, 2013; Paudel and Sanfaçon, 2018; Wu and Ye, 2020). Although symptomless, SPPV persistence in the cells could induce constitutive activation of defence genes, which is costly to the plant's fitness (Igari et al., 2008; Pumplin and Voinnet, 2013; Bradamante et al., 2021).

The low SPPV concentration means the virus expression was reduced, probably due to symbiosis, recovery or both. Recovered tissues are resistant to re-infection by cognate virus(es) because of cross-protection, whereby pre-infected plants are primed for RNAi defence by complementary vsiRNA (Loebenstein and Carr, 2006; Mwanga et al., 2013; Paudel and Sanfaçon, 2018; Bradamante et al., 2021). Our results and analysis of SPPV are consistent with Kreuze et al., (2020).

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; Katz et al., 1998; Loebenstein and Carr, 2006). Systemic priming of AGO2 was induced through SAR by cucumber mosaic virus in resistant Arabidopsis (Ando et al., 2021).

6.5. **DEGs responsive to viruses**

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. JA and 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).

Viruses can arrest the cell cycle and insert their genetic material for multiplication with the plant genome (Hanley-Bowdoin et al., 2013). Cyclin D3:1 increases mitotic cell cycles and growth by promoting G1/S transitions (Menges et al., 2006). The gene was highly downregulated in the stunted ME_1 and upregulated in TJ_1. Cyclin-dependent kinase B2;2 was downregulated in ME_1 and upregulated in TJ_1. It regulates gene expression and cell division through JA and SA signalling (Li et al., 2018).

Viruses hijack host translation machinery by interacting with cofactors that facilitate their replication and movement. Cofactors are mostly susceptibility proteins, and their inhibition or mutation can lead to recessive resistance against viruses that require them (Carr et al., 2010; Nicaise, 2014; Hashimoto et al., 2016). One of the most common genes responsible for recessive resistance is eIF4E, which limits translation rate. It was upregulated in TJ_1 but downregulated in ME_1. Viruses often target the translation machinery to control translation of their own and host genes. Availability of mutants or variants of translation machinery genes or cofactors inhibits virus accumulation without inducing typical disease resistance responses, thus recessive resistance (Carr et al., 2010; Hashimoto et al., 2016).

6.6. **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:

a) Nodulin MtN21/EamA-like transporter family protein transporting amino acids (such as glutamine and histidine) and auxins in the vascular tissue (Vanholme et al., 2010; Kan et al., 2017).
b) 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).
c) Metallothionein 2A scavenging of ROS to reduce oxidative stress that could damage the infected cells (Patankar et al., 2019).
d) Strengthening the cell wall through lignification by cinnamoyl-CoA reductase (CCR)-like gene (Bart et al., 2010)

6.7. **KEGG pathways**

Enriched KEGG pathways include vitamins and phenolic compounds which are antioxidants that modulate ROS in infected cells. Expression of antioxidant genes is influenced by the circadian rhythm (Dutilleul et al., 2003). 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).

Sucrose metabolism and phenylpropanoid biosynthesis products 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 (Proels and Hückelhoven, 2014; Singh and Singh, 2018).

MAPKs are activated by and respond to diverse biotic and abiotic stimuli through crosstalk with phytohormones JA, SA, ABA, ET and IAA. 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).

6.8. **SPPV-SPCSV resistance mechanism**

Virus inhibition in TJ was consistent with resistance (Tavantzis, 1984; Karyeija et al., 1998). We hypothesize 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; Almagro et al., 2009; Carr et al., 2010; Zvereva and Pooggin, 2012; Kidwai et al., 2020). Recessive resistance 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). Although commonly associated with eIF4E, other recessive resistance genes have been reported, such as *rwm1* gene against water melon mosaic virus (Nicaise, 2014; Ouibrahim et al., 2014; Hashimoto et al., 2016). Functional genomics analysis will help to understand the resistance mechanism and verify the suggested putative resistance genes.

7. CONCLUSIONS 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. 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 viruses in five of six sweet potato cultivars from a producer farmer and Hungarian gene bank. 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. 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'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. Individually, 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. Eighteen PT sweet potatoes obtained from CIP in Nairobi, Kenya and two from our collection in Gödöllő, Hungary, were grafted to the SPPV-SPCSV infected plant labelled *A6.1*. Cultivar Melinda, Mugande and *Ylw* developed virus symptoms in the growth chamber from five wpi. They also had high virus titres and 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 higher in ME than in TJ. Tio Joe was therefore considered resistant to SPPV-SPCSV 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.

8. 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 characterization 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.

9. SUMMARY

Sweet potato is a highly fibrous and nutritious crop with pharmaceutical and ornamental values. It is the third most important root and tuber crop globally and one of the most essential staples in Sub-Saharan Africa. Its global production has been on the decline in the past decade. The crop is affected by over 30 viruses globally. To avoid yield losses, propagation materials must be virus-free as viruses accumulate in successive planting seasons in the fields leading to cultivar degeneration. Viruses in sweet potatoes are transmitted mainly through vegetative propagation and by aphids and whiteflies. Severity of symptoms varies with plant genotype, age, environment and interactions between the viruses that infect it. Synergistic virus interactions are more damaging than single infections.

Conventional sweet potato breeding is difficult because of its hexaploidy and heterozygosity (2n=6x=90). Resistance breeding has been successful in controlled environments and against SPFMV. CRISPR-Cas is promising in developing resistant cultivars to virus diseases since resistance to SPVD was increased through CRISPR-Cas13 targeting of SPSCV-RNase3.

Sweet potato has been cultivated in Hungary for at least four decades, with commercial production increasing as the nutritious roots become popular with consumers. We collected sweet potatoes from farmers' fields, researchers, retail stores and the national gene bank of Hungary to check for viruses. We detected seven viruses after testing 110 plants from seven regions in Hungary for 15 viruses from nine genera. These were five RNA viruses: SPCSV, SPFMV, SPV2, SPVC and SPVG and two DNA viruses, SPLCV and SPPV. This study was the first to report sweet potato virus disease (SPVD), SPCSV, SPPV and SPLCV infecting sweet potatoes in Hungary.

Due to the increasing sweet potato cultivation in Hungary, viral diseases were predicted to increase. Therefore, there is an urgent need to produce propagation materials that are pathogen tested to save farmers from yield and profit losses. However, there's no evidence of regulation on virus-free sweet potato production or planting in Hungary. Free movement of virus infected germplasm in the European Union poses significant phytosanitary risks. Through heat treatment and meristem tip culture, we removed five viruses: SPFMV, SPV2, SPVC, SPVG and SPLCV from five local sweet potato cultivars labelled *T96, 92R, 12R* from Hungarian gene bank and *Blk* and *Ylw* from a producer farmer. Hopefully, this work will provide an impetus for setting up a system for producing PT sweet potato propagation materials for Hungary and the sweet potato growing regions of Europe.

Our results are consistent with other studies that showed SPPV was widespread in sweet potato germplasm globally, yet it was recalcitrant to heat treatment. SPCSV is the most damaging virus in sweet potatoes as it synergizes with other viruses causing severe diseases and yield losses. SPPV and SPCSV do not cause much damage in single infections. So, when we found a plant in a farmer's field in Hungary with severe disease symptoms, yet it contained only SPPV and SPCSV, we decided to examine the mechanism of their co-infection. After screening for resistance to SPPV-SPCSV in 20 PT sweet potato cultivars using symptoms and virus titres, Tio Joe was found to be resistant, while Melinda, Mugande and *Ylw* were susceptible. HTS and transcriptome analysis of the resistant Tio Joe and susceptible Melinda pointed towards systemic acquired resistance and recessive resistance as possible mechanisms responsible for the

SPPV-SPCSV resistance. Putative resistance genes that were identified and validated were nodulin MtN21 /EamA-like transporter family protein, SAUR-like auxin-responsive protein family, metallothionein 2A and cinnamoyl-CoA reductase (CCR)-like gene. The role of these genes in SPPV-SPCSV resistance should be verified through functional genomics analysis to help understand the resistance mechanism.

This study emphasized the importance of planting virus-free sweet potatoes and recommended strict regulation and comprehensive investigation of their distribution and economic impact in Europe.

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12. APPENDICES

12.1. Bibliography (List of works consulted)

12.1.1. Peer-reviewed articles with impact factor

- Kiemo, Francis Wanjohi, Toth, Z., Salamon, P., and Szabo, Z. (2022): First Report of Sweet Potato Chlorotic Stunt Virus Infecting Sweet Potatoes in Hungary, Plant Disease, 106(2), 773. https://doi.org/10.1094/PDIS-05-21-0944-PDN
  Q1, Impact factor: 4.614
- Kiemo, Francis Wanjohi, Salamon, P., Jewehan, A., Tóth, Z., and Szabó, Z. (2022): Detection and elimination of viruses infecting sweet potatoes in Hungary, Plant Pathology, 71(4), 1001–1009. https://doi.org/10.1111/ppa.13519
  D1, Impact factor: 2.772
- Jewehan, A., Kiemo, F. W., Salem, N., Tóth, Z., Salamon, P., and Szabó, Z. (2022): Isolation and molecular characterisation of a tomato brown rugose fruit virus mutant breaking the tobamovirus resistance found in wild Solanum species, Archives of Virology, 167(7), 1559–1563. https://doi.org/10.1007/s00705-022-05438-2
  Q2, Impact factor: 2.685

12.1.2. Conference presentations

- Kiemo, F. W. (2022): Detection and elimination of viruses infecting sweet potatoes in Hungary, in Bánfalvi, Z., Gócza, E., Olasz, F., Pál, M., Posta, K., and Várallyay, É. (eds) 5th National Conference of Young Biotechnologists : Program and abstracts, 35
- Kiemo, F. W., Ongoya, G., and Szabó, Z. (2021): Yield performance of 44 sweet potato varieties in Kirinyaga county of Kenya, in 3rd Annual International Research Conference : Book of Abstracts, 76–77

12.2. **Primers used**

12.2.1. **Table 1**. Primers used to detect viruses through PCR and qPCR

Genus	Virus	Primer	F (5'-3')	R (5'-3')	Product size (bp)
Crinivirus[e]	Sweet potato chlorotic stunt virus (SPCSV) – West African strain	CH2N	CGTCGACACTTGTTGCGGTA	GCCCAATACACCGGATGTGAC	194
		CL43U[b] CL43L[b]	ATCGGCGTATGTTGGTGGTA	GCAGCAGAAGGCTCGTTTAT	486
	Sweet potato chlorotic stunt virus (SPCSV) – East African strain	H5[b] CL43L[b]	TTGGTGGTACGATGAAGGTCC	GCAGCAGAAGGCTCGTTTAT	475
Potyvirus[e]	Sweet potato feathery mottle virus (SPFMV)	SPFMV5	ATGCGAAACCGTACAAAGG	TCAGTTGTCGTGTGCCTCTC	243
	Sweet potato virus 2 (SPV2)	SPV2 _2	GCCAAGGTATGGCCTACAGA	CGGGACTGAAAGACACGAAT	331
	Sweet potato virus C (SPVC)	SPVC3	ACACGCTCAACTCAGGAACAG	GCATATCGCGCAAGACTCA	350
	Sweet potato virus G (SPVG)	SPVG3	TGCAGCCTCAAAACCAC	AACCGTACCAGCATTCACATC	229
	Sweet potato mild speckling virus (SPMSV)	SPMSV1	GCCAAAACCAACAAGCATCA	ATTCGCATTTCCTCATCATCT	276
		SPMSV2	GTGCGCCAAGTCATGGA	TGAGTATGAAGCGTCGCG	125
		MastvKF[d] MastvsR[d]	GACAGACCCCTAGGGTGA	ACTGCATATAGTACATGCCACA	436
	Sweet potato latent virus (SPLV)	SPLV1	GGGTGATGATGGACGGAGACA	CCGATGATGTGTATTTGTGAGC	299
Ipomovirus[e]	Sweet potato mild mottle virus (SPMMV)	SPMMV3	GTTGTGGCACTTGAACCAGG	AGGCGACAAGTTACCTAGC	227
Cucumovirus[e]	Cucumber mosaic virus (CMV)	CP2	TCCTGCCTCCTCGGAC	TGGGAATGCGTTGGTGCT	268
Carlavirus[e]	Sweet potato virus C-6 (SPVC6)	SPVC6_10	CAGGCGTTATTGGGGGTAG	GCTCGTTGGCTCTTGAATAGG	290
	Sweet potato chlorotic fleck virus (SPCFV)	SPCFV1	GHATHGCTAGRCCYCCRAAT	TCACAYYTCTTRCCACACTCATA	398
Solendovirus[f]	Sweet potato vein clearing virus (SPVCV)	SOLN_RT1	TCCAAGGYTACAATTGGTTTTC	TCTCCCAGGGGCATTCT	158
Cavemovirus[f]	Sweet potato collusive virus (SPCV)	CAVN RT 1	GGGARAAACATAAAACMTAYGC	CCTTGYGGTACTGTAAATGC	421
Begomovirus[f]	Sweet potato leaf curl virus (SPLCV)	SPGN15[c]	CCCCDGTGCGTRAATCCAT	ATCCVAAYWTYCAGRGAGCTAA	935
Badnavirus[f]	Sweet potato pakakuy virus (SPPV)	BKS1N[d]	CAAAYTAGGAGGCAGATAAATG	GGTCTYCTKAYGTTCCACCTT	867
		SPPV_CP2	CAGCAGTCACAGAAGTCGG	CCTTTCCAGCGTCCTTCTG	173

[b] Primers reported in Alicai et al., (1999).

[c, d] Primers modified from Li et al., (2004) and Mbanzibwa et al., (2011), respectively.

[e] RNA virus

[f] DNA virus

12.2.2. **Table 2**. PCR primers for amplification of sweet potato chlorotic stunt virus (SPCSV) complete genome

	Primer	F (5'-3')	R (5'-3')	Genome target region	Product size (bp)
RNA 1	Ch8, Ch19	GAAATACTTCCAGCTATCCAAATTTGGTG	GCCGCACTGGGTTTCAA	1 to 4585	4585
	Ch21	CAGACGGTAATAGCGAAGG	ACCTTCTAAATCTAACAATAAGAAAAGGA	4095 to 8442	4348
	Ch22, Ch20	TCCTTTTCTTATTGTTAGATTTAGAAGGT	AACCTAGTTATTTAAATACTAGGTTTTCC	8414 to 8637	224
RNA 2	Ch_start 24	GAAATACTACCCAGGTTTTTCCATGAG	TACCACCAACATACGCCGAT	1 to 945	945
	Chong_1 19	TGGGCCGGGAGTAATCAA	CTCGTCCGGTTCGTCTG	316 to 4193	3878
	Chong_2 20	AGAGCGCGTGTAATGTTCAAGATA	ATACTAGGTTTTCCAAGGTCCAT	3894 to 8092	4199
	Ch_end 25	AGGATCGCAATGGCTAA	GGCCTAGTTATTTAAATACTAGGT	7790 to 8107	318

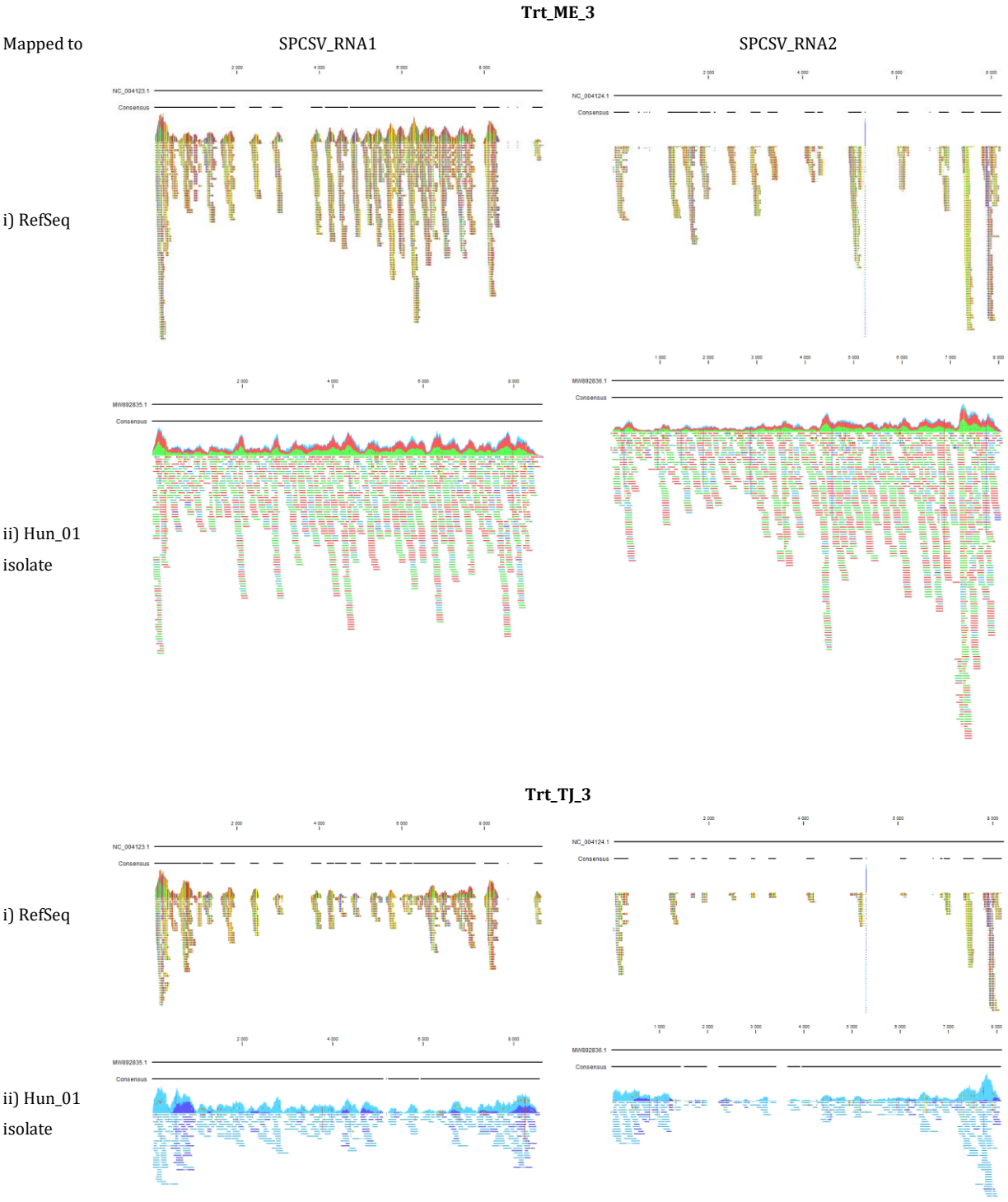
12.2.3. **Table 3**. Primers used to validate RNA-Seq results by qPCR and for RNAi genes expression analysis

Gene	Gene/Primer ID	F (5'-3')	R (5'-3')	Product size (bp)
CCR-like	itf05g00780	CGATGGCCTCTGTCTTTCCA	GCCTCTCCAAGAAATACCGTCT	187
White-brown complex homolog protein	itf09g03330	CCAAGCAGGAAGGAGAAGTGC	GGCGCAGCCAATAGTAGCC	123
Putative mitochondrial RNA helicase	itf09g08260	CGAACAGGACGTGCAGGC	CCTTGGGAGCTCAATAAATTTGCA	117
Nodulin mtn21 /Eama-like transporter family protein	itf09g10970	GGGGCCTGTCTTTGTCACA	CCAGCAACACTTCCAACAAAGA	105
Pathogenesis-related	itf09g23200	ACTGGGATTATAGGACGGCG	TATTGGTAACCCTCAAACATCTGC	153
Saur-like auxin-responsive protein family	itf09g23650	ACTTTGCAGTTTATGTGGGCG	CCGAACTCTTCCTCAGCCTG	112
Metallothionein 2a	itf07g19700	AGTGACTGCGCGTGTGAA	CAAGGGTCACAGGTGCAGT	148
DCL2 [a]	2_DCL2	TCCCCAAGGTGCTAGGAG	CTCGTTCAGCTCTCTCGC	162
DCL4 [a]	2_DCL4	TGCGAACTGTTGGAAGCCT	GGAACCAAAGCGCCCCT	188
AGO1 [a]	3_Ago1	GTTGCTTCTCAGGATTGG	GCAATTCTTTGATCATGCCAC	148
AGO4 [a]	4_Ago4	CTGCCATCTCAGTTGTTGCC	TGGACACTTTCTCCTCCAGCT	177
SDE5 [a]	2-SDE5	CGTCTCGAGATTCTGCTGGA	CCTTGAAGGCCGAACTGC	167
Actin [b]	swt-actin	TTCTCCTTTCTAACACTCCTCAG	CGCCTCGCTCTCTCTAGATCC	60

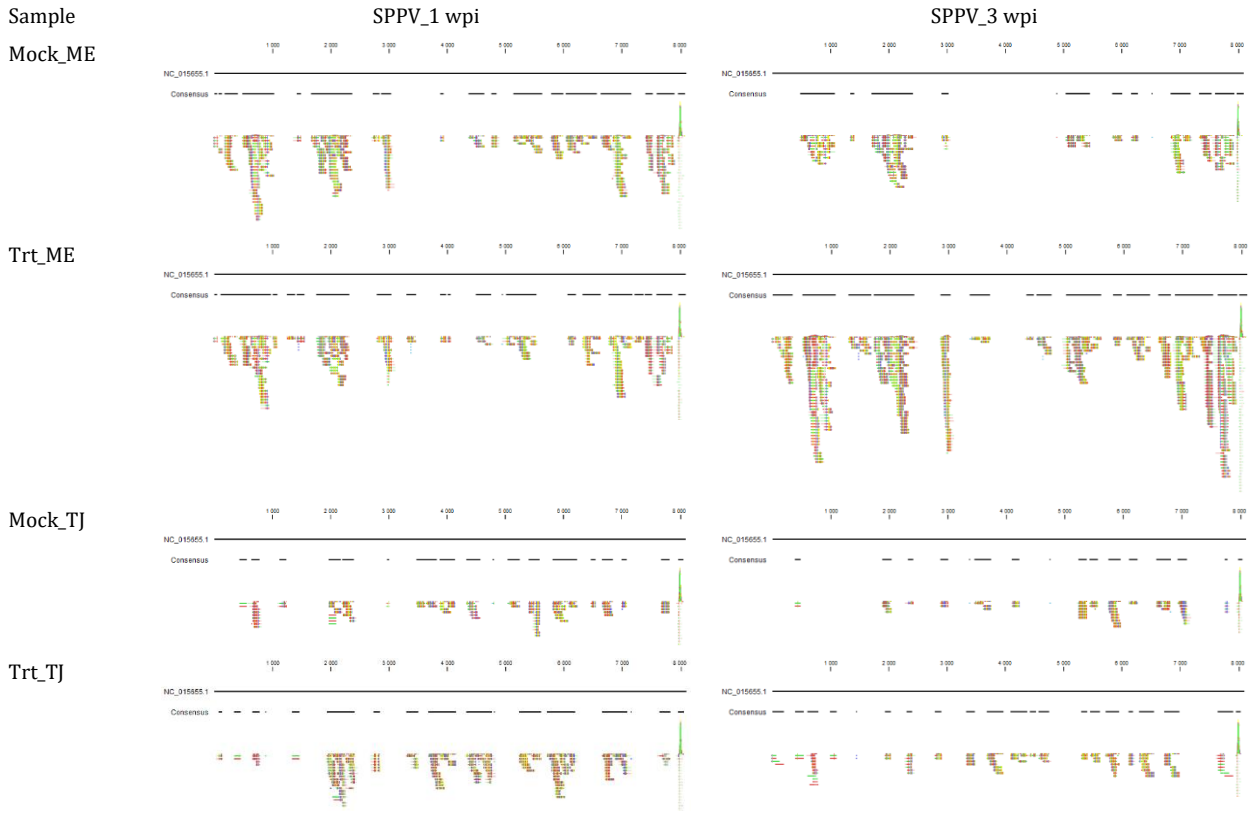
[a] Gene involved in RNAi.

[b] Primer reported in Kreuze et al., (2020)

12.3. RNA-Seq reads mapping to SPCSV and SPPV



(a)



(b)

Figure 1. Mapping of SPCSV reads at three wpi to the reference genome and Hun_01 isolate. SPCSV was not detected in the mocks (a). Mapping of SPPV reads one and three wpi to the reference genome. SPPV was detected in all mocks and treatments (b).

12.4. Experiments flow chart

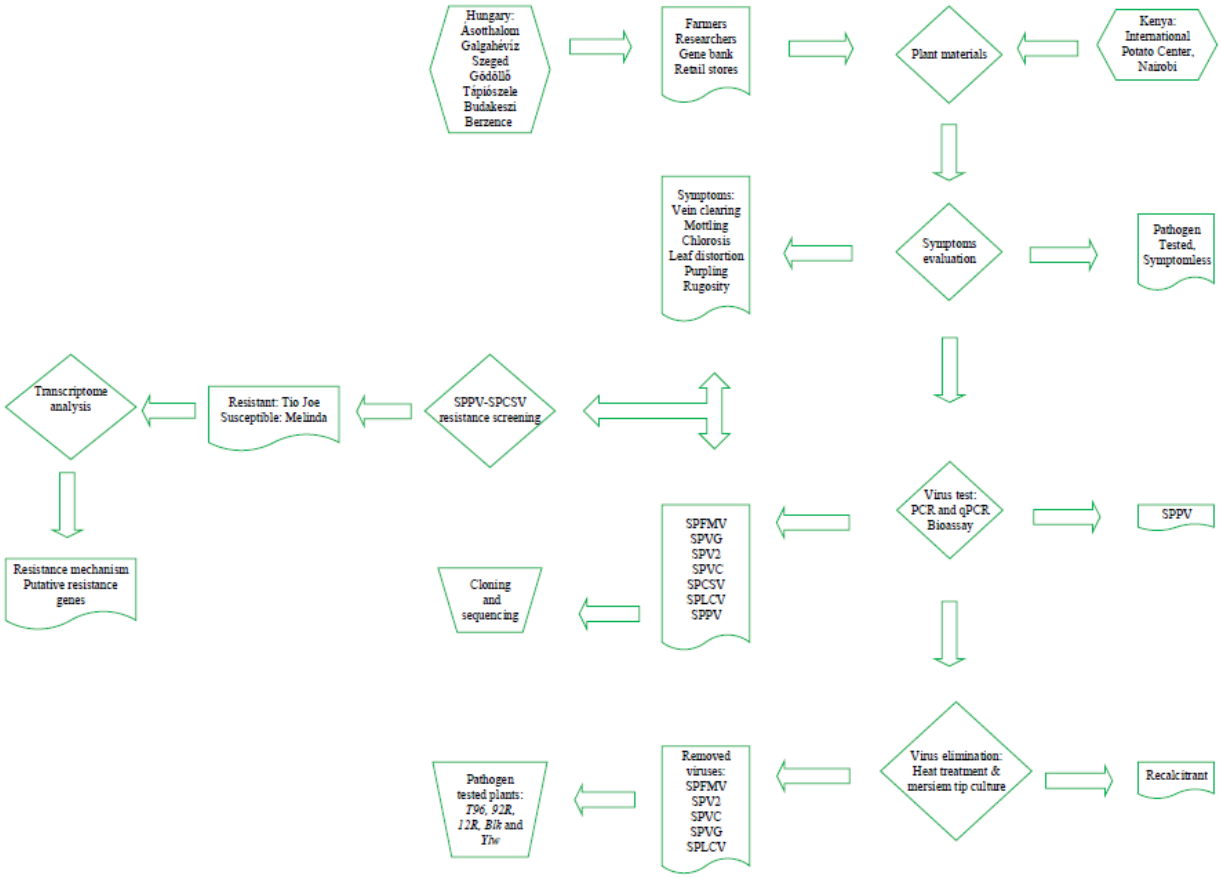


Figure 2. Flow diagram showing the connections between the experiments conducted.