Characterization of Cassava brown streak virus proteins draw their sides during mixed infections and reveal P1 as a silencing suppressor

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Abstract

Cassava brown streak disease (CBSD) is currently one of the major constraints on cassava production in Africa. CBSD is estimated to cause annual economic losses of over $100 million USD. CBSD is caused by at least two viral species: Cassava brown streak virus (CBSV) and the Uganda cassava brown streak virus (UCBSV). In field, CBSV and UCBSV occur in single and mixed infections with the potential to be found in mixed infections with other viruses. The interactions between CBSV and other viruses are poorly understood and many functions of CBSV genes are not fully characterised. In this study we analysed the interaction of CBSV with non-related viruses, for potential synergistic interactions, namely tobacco mosaic virus (TMV), and potato virus Y (PVY), both very well characterised for their infection and symptomatology in *Nicotiana* species. These interactions demonstrated to be synergistic with TMV and antagonistic with PVY. Then P1, P3, 6k1, CI, 6k2, VPg, NIa, NIb, Ham1-like and CP from CBSV were analysed separately, to determine which genes from CBSV were responsible for the direction of these interactions. For this analysis, transgenic lines expressing single CBSV genes were used, providing information about the importance of Ham1. Further functional analysis of these CBSV genes was carried out, analysing silencing suppression activity through agroinfiltration assays. This confirmed silencing suppression activity for the CBSV P1 protein and demonstrated that a functional LRRA domain is required for this activity.

Introduction

Cassava is the third most important source of carbohydrates in the tropics, after grain crops (Clifton and Keogh, 2015). It is estimated to feed approximately 800 million people in the tropics and subtropics, playing a key role in the diets of most sub-Saharan countries in Africa (Alicai et al., 2016; Fessenden, 2014; Gomes and Nassar, 2013). Currently the major production of cassava takes place in sub-Saharan Africa (Food and Agriculture Organisation Statistics Database, 2018). This crop is mainly propagated by stem cuttings, which has an impact on the transmission of diseases caused by bacteria, fungi or viruses (Taylor et al., 2012; Yadav et al., 2011).Cassava brown streak disease (CBSD) has become a major concern for cassava producers, replacing cassava mosaic disease (CMD) as the most important viral disease in East and Central Africa (Legg et al., 2011; Patil et al., 2015; Tomlinson et al., 2018). CBSD used to be restricted to East Africa, now it has been found in Central Africa including Burundi, Rwanda and eastern Democratic Republic of the Congo (Casinga et al., 2020). CBSD is caused by at least two viral species: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus,* (UCBSV), both in the *Ipomovirus* genus of the *Potyviridae* family (Monger et al., 2001b, 2001a; Tomlinson et al., 2018). CBSD symptoms can vary widely depending on the environmental conditions, the strain of the virus and the susceptibility of the cassava variety (Mohammed et al., 2012; Tomlinson et al., 2018; Winter et al., 2010), but typically causes circular chlorotic blotches or feathery chlorosis along the vein margins of the leaves and necrosis of the starchy roots (Mohammed et al., 2012; Nichols, 1950; Winter et al., 2010). Necrosis in roots can reduce the amount of starch storage up to 65% (Nuwamanya et al., 2015),

this reduces the yield and quality of the crop, resulting in economic losses up to $100 million USD annually in areas where the disease has been reported (Mohammed et al., 2012; Tomlinson et al., 2018).

Interactions of CBSV co-infecting the same host with UCBSV has been previously reported (Ogwok et al., 2015), while interaction with African cassava mosaic virus (ACMV), that produce CMD, has not been proved to occur in the field, but it has been only tested under laboratory conditions (Ogwok et al., 2010). Several studies suggest that infections in wild and crop plants are commonly produced by the interaction of two or more viral species, demonstrating that virus coinfections seem to be the rule rather than the exception (DaPalma et al., 2010; Mascia and Gallitelli, 2016). Latham and Wilson (2008) mentioned the importance of potential trans complementation occurring with viruses and potential partners, the outcome from these viral coinfections can be studied and replicated performing trans complementation assays, using plants that transiently or constitutively express viral proteins from the tested virus, then transgenic and wild type plants can be infected and the differences can be quantified by changes in viral accumulation, symptomatology, host range, vector transmission, cell to cell movement and systemic movement. Potyviruses often demonstrate their ability to synergise other viral infections (Syller, 2012). For CBSV there are few studies where investigate its potential interactions with other viruses. In the field, CBSV and UCBSV are suggested to act competitively during mixed infections in cassava, as CBSV and UCBSV loads are observed to decline when both viruses are present. In some cases titres of UCBSV, only, are reduced during mixed infections, displaying a large difference to CBSV loads (Abarshi et al., 2012; Kaweesi et al., 2014; Mbanzibwa et al., 2011; Ogwok et al., 2015; Otti et al., 2016). Under laboratory conditions mixed infections have been tested co-infecting of *Nicotiana benthamiana* with ACMV and UCBSV, increasing the severity of symptoms and resulting in the death of the plants (Ogwok et al., 2010).

The genome of CBSV is a positive-sense single-stranded RNAs, that encodes a polyprotein which later is processed into 11 mature proteins. In this study we performed assays to determine whether CBSV was able to interact with other viruses in an antagonistic or synergistic manner, choosing unrelated but very well characterised model viruses, TMV and PVY, as viral partners for interactions. Following this, 10 of the 11 individual CBSV genes were tested to identify which of them were responsible for these interactions.

To date CBSV gene functions have not been fully characterised and are usually inferred, based on homology with other members of the *Potyviridae*. CBSV and its closest relative, UCBSV, exhibit unusual features in their genomes, including the absence of coding sequence for the Helper component proteinase (HC-Pro), a single copy of the P1 protein and an unusual Ham1-like open reading frame, which shares homology with the Maf/Ham1 from yeast, member of the inosine triphosphate pyrophosphatase (ITPase)-like proteins (Mbanzibwa et al., 2009; Tomlinson et al., 2019a).

Synergistic interactions may be attributed to functions associated with movement proteins, coat proteins, replicases or suppressors of gene silencing (Bowman Vance et al., 1995; Latham and Wilson, 2008; Pruss et al., 1997; Syller, 2012). In UCBSV; P1, P3 and Ham1 proteins have been analysed previously for suppression of gene silencing activity, showing that P1 acts as a silencing-suppressor (Mbanzibwa et al., 2009), while P1 or any other gene in CBSV have not been investigated for their role as silencing-

suppressor. In other potyviruses, HC-Pro, P1b and P1 have all been identified as silencing-suppressor proteins that interfere with the assembly of RISC, by binding siRNA or interacting with the RISC Argonaut protein (Giner et al., 2010; Lakatos et al., 2006; Mérai et al., 2006; Valli et al., 2008). For UCBSV, P1 the presence of the conserved LRRA domain was suggested to be homologous to the reported LxKA motif in P1b of cucumber vein yellowing virus (CVYV) (Mbanzibwa et al., 2009). The conserved LxKA motif and the putative zinc finger in P1b are essential components for silencing-suppression activity in the ipomovirus CVYV (Valli et al., 2011, 2008).

Recently, some functions have been attributed to the CBSV Ham1-like protein. Tomlinson et al. (2019a) showed that it acts as a determinant of necrosis but is not essential for viral infection, showing that the virus can replicate, move and develop symptoms when CBSV Ham1 is modified or removed from the CBSV infectious clone. Its ITPase activity was confirmed in the presence of non-canonical XTP and dITP, using *in vitro* assays (Tomlinson et al., 2019a). Additionally two other theorical functions have been proposed to this protein, such as disruption of plant signalling pathway and reduction of ITP antagonistic/agonistic interactions with nucleotide binding proteins (James et al., 2021). Tomlinson et al. (2019b) also demonstrated that coat protein (CP) from CBSV is associated with early presence of systemic necrosis and severity of symptoms.

Results

Assessment for synergism by CBSV during mixed infections with unrelated viruses.

This study aimed to identify the role of CBSV genes during mixed infections, then CBSV was examined for its ability to interact with other viruses in a synergistic or antagonistic manner. Then, interactions with unrelated, but well characterised model viruses tobamovirus (TMV) and potyvirus (PVY^{O}), were arranged in combinations as follow: CBSV/TMV and CBSV/PVY. Each combination and single viruses were inoculated into *N. benthamiana* and *N. tabacum*. Single TMV infections displayed the characteristic mosaics patterns of dark and light greens, yellow spotting and malformation of leaves in *N. tabacum,* whilst the same infection displays stunting, mosaics, yellow streaks on leaves and curling leaves in *N. benthamiana*. Typical symptoms for PVY^{O} are mottling and mosaic symptoms on leaves in *N. tabacum* and stunting, yellow patches on leaves and curling leaves in *N. benthamiana*. The CBSV_Tanza IC does not produce severe symptoms in *N. tabacum,* but it can produce yellow spotting on the *N. benthamiana* leaves, necrotic streaks close to the veins, stunting, curling leaves and kill the entire plant at 20 days post inoculation (DPI).

For the first interaction, CBSV/TMV mixed infection displayed slight stronger symptoms in both types of plants in comparison to the single TMV infection. *N. benthamiana* showed a fast-systemic necrosis in mixed infections in comparison to the single TMV infection (Figure 1A), whilst *N. tabacum* showed a faster symptomatology with mosaic and necrotic lesions in comparison to the single TMV infection (Figure 1C). TMV viral titres showed an early enhancement of viral accumulation in mixed infections, compared to single infections in both *N. benthamiana* and *N. tabacum* (Figure 1B-1D), Wilcoxon test

indicated that increment in viral titre differ from single infections at 6, 9, 15, 18 and 21 DPI in *N. benthamiana*, while differences appear at 6, 18 and 21 DPI in *N. tabacum*. This indicates that CBSV could interact with TMV in a synergistic manner.

For the second interaction, CBSV/PVY, mixed infections showed a reduction in symptoms in *N. benthamiana* and *N. tabacum*. *N. benthamiana* infections were characterized by the absence of crinkle symptoms, a typical symptom for PVY, and the absence of the systemic necrosis that is usually present during CBSV infection (Figure 1E). In *N. tabacum,* mixed infections showed a slight reduction of mottling in comparison to PVY infection (Figure 1G). Quantification of PVY titres showed a trend of reduction in PVY accumulation during mixed infections in comparison to single infections in *N. benthamiana,* but an increase in *N. tabacum.* Wilcoxon test indicated that differences due to the reduction of PVY titre were present at 12, 15, 21 and 27 DPI in *N. benthamiana* while no reduction in PVY titre was observed from mixed infection in comparison to single infection in *N. tabacum* (Figure 1F-1H).

[Figure 1. All figures and tables, including legends

are present in a different file per image]

Identification of CBSV genes responsible for viral interactions.

Transgenic *N. tabacum* lines were generated for each of the individual CBSV encoded genes, the expression of CBSV transgenes in plants were verified by RT-PCR (Figure 2) and Q-RT-PCR (Table S1). Then these transgenic lines were mechanically inoculated with TMV or PVY to identify which CBSV proteins might interact synergistically or antagonistically with TMV or PVY. Healthy plants expressing P1 from CBSV showed an atypical phenotype, developing new leaves smaller and slower than the rest of transformed plants, making this line unsuitable for subsequent infection assays.

[Figure 2]

For plants infected with TMV, viral titre tend to be higher at early stages of the infection in plants expressing P3, CI, 6K2, VPg, NIb and Ham1 from CBSV (Table S2). A Kruskal-Wallis test, per day analysed, showed that the differences in these transgenic lines in comparison to the wild type plants were significant, predominantly at 6 and 14 DPI, and Dunn test showed that only lines 6K2, VPg, Ham1 and NIa had significant differences against the wild type *N. tabacum* over the time, however NIa did not induce an enhancement of TMV accumulation (Table 1,Figure 3).

[Table 1]

[Figure 3]

During PVY infection, transgenic lines of *N. tabacum* expressing the P3, 6k2, NIb and Ham1-like genes from CBSV showed a reduction of symptoms and their PVY titre tend to be lower than wild type plants (Figure 4, Table S3). Differences of PVY titre were analysed performing a Kruskal-Wallis test and then by

Dunn test, which indicated that the reduction in PVY titre in plants expressing P3, 6k2, Nlb and Ham1-like protein was significant, mainly at the 10th DPI and 26th DPI(Table 1, Figure 4).

[Figure 4]

Identification of CBSV silencing suppression activity

While some CBSV genes where able to enhance viral infection, by increasing viral accumulation, the expression of P1 from CBSV in *N. tabacum* spoiled the development of the transgenic plants where P1 was expressed. Therefore, a functional analysis for silencing suppression activity was performed by agroinfiltration assays, using the 16c *N. benthamiana* line. Expression vectors encoding individual CBSV genes and GFP expression vectors were co-infiltrated into plant leaves. In the absence of a suppressor of gene silencing, GFP transcripts are degraded, but if the gene silencing mechanism is suppressed GFP can be transiently overexpressed in the infiltrated area. Hence, gene silencing suppression was examined at 5 days post-inoculation (DPI). Plants were assessed for expression of GFP within the agro-infiltrated zone, using a trans-illuminator and a fluorescence stereomicroscope.

Of the 10 CBSV genes that were screened during co-agroinfiltration assays, P1 was the only protein that exhibited a detectable gene silencing-suppression activity (Figure 5). While the other 9 genes, screened at this work, displayed only a dimmed impression as consequence of the agroinfiltration damage with no discernible GFP, indicating no silencing suppression activity.

[Figure 5]

The P1 LRRA domain is essential for silencing-suppression activity.

Based on similarity to other viral silencing-suppressors, Mbanzibwa et al. (2009) suggested the conserved zinc-finger and the LRRA domain from UCBSV P1 were likely to be involved in suppression of gene silencing, but this was not investigated. Having confirmed that CBSV P1 could suppress gene silencing, the P1 region of CBSV was modified in the available infectious clone from the Tanza strain, modifying the LRRA domain to encode the LAAA, this modified P1 region was then transferred into the pCambia2300_EC expression cassette for transient expression. Assessment of silencing-suppression activity for the mutant P1, demonstrated that mutation of the conserved LRRA domain to LAAA abolished silencing suppression as no GFP was discernible (Figure 6).

[Figure 6]

The P1 LRRA motif is important for infection

The modified CBSV_Tanza_P1_LAAA infectious clone was then inoculated onto *N. benthamiana* to assess its virulence. Whilst the unmodified CBSV_Tanza IC infections developed characteristic stunting of the plant-growth, followed by necrosis of shoot tips and then collapse of the plant, infections with the modified CBSV_Tanza_P1_LAAA failed to develop any apparent symptoms. Analysis by both RT-PCR and

TAS-ELISA failed to detect systemic infection at any upper leaves in the plants infected with the LAAA-modified infectious clones, suggesting that LRRA domain in P1 is essential for CBSV life cycle in *N. benthamiana* (Figure 7).

[Figure 7]

Discussion

CBSV and UCBSV have genome structures that are atypical for the *Potyviridae*, so inference of viral protein functions has been difficult to make. Previously, Mbanzibwa et al. (2009) showed that UCBSV P1 has activity as a suppressor of gene silencing and suggested that Ham1 could prevent incorporation of non-canonical nucleoside triphosphates (NTPs) as Ham1 from yeast does. Tomlinson et al. (2019a) showed that CBSV Ham1 does not protect yeast cells from the incorporation of non-canonical NTPs when it is overexpressed in yeast as demonstrated by Ham1 from yeast, neither it could reduce mutation rate in the viral genome of CBSV. Tomlinson et al. (2019a), however confirmed that CBSV Ham1-like has ITPase activity during *in vitro* assays, additionally they showed that it is a determinant of necrosis during CBSV infection in *N. benthamiana*. Here we expand on the knowledge about functionality of the proteins encoded by CBSV. We identified that like P1 from UCBSV, the CBSV P1 also functions as a gene silencing suppressor and demonstrate that this activity requires a functional LRRA domain. Coupled with this, we found that CBSV can act synergistically with non-related viruses such as TMV, but can act antagonistically with other viruses such as PVY, in this case these two model viruses were used not for their possible interaction in the field with CBSV, but because their infection and symptomatology are very well characterised in *N. tabacum*. For CBSV, genes have not been experimentally scrutinised for their ability to support an interaction with another virus. Here showed the effect of several CBSV genes using two unrelated viruses, even though they have contrasting outcomes.

Synergistic interactions involving CBSV

CBSV and UCBSV occur in geographically overlapping areas (Ogwok et al., 2015), where mixed infections have been reported to occur, mainly in susceptible varieties of cassava. In this study CBSV was analysed for possible synergistic effect with unrelated, but very well characterized viruses TMV and PVY. Whilst synergism was observed with TMV, antagonism was found with PVY. Otti et al. (2016) suggests that interactions between the CBSD viruses could be synergistic due to the presence of higher CBSV titre in the presence of UCBSV, although Kaweesi et al. (2014) also suggested that these interactions are rather competitive, having a higher titre for CBSV than for UCBSV. For interactions between CBSD viruses and ACMV, information is limited to the artificial synergism observed in laboratory conditions in *N. benthamiana* (Ogwok et al., 2010). The potential of any synergistic interaction between CBSD viruses and any other virus have not been described in the field; however, the impact and the frequencies of these interactions remain unknown for cassava and warrant further investigation.

TMV and PVY infection in CBSV transgenic lines

The simulated interactions with CBSV, TMV and PVY were planned to identify which viral genes could play a role during synergism or antagonism, when any interaction is present. Some transgenic lines expressing CBSV proteins showed an enhancement of TMV viral titre and symptoms at early stages of the infection, these plants were expressing the VPg, 6k2, NIb and Ham1-like form CBSV. Where the mechanism is known, usually synergism can be attributed to functions associated with movement proteins, coat proteins, replicases or suppressors of gene silencing (Bowman Vance et al., 1995; Latham and Wilson, 2008; Pruss et al., 1997; Syller, 2012).

In other potiviruses, P3 has been previously reported to be implicated in synergistic interactions; P1, HC-Pro and P3 from TEV are able to enhance PVX infections (Pruss et al., 1997). In addition, the P3N-PIPO protein, which is derived from the ribosomal frame shifting at the N-terminus of P3, from Clover yellow vein virus (CYVV), has been reported to act synergistically during infections with White clover mosaic virus (WCMV) in pea (*Pisum sativum*) (Hisa et al., 2014). This synergism has been suggested to be a consequence of the cell-to-cell movement enhancing activity of PIPO, which facilitates spread of the synergised viral genome (Vijayapalani et al., 2012).

CI and 6k2 in potiviruses have not been reported to enhance other viral infections. Functions of these two proteins are directly involved with viral replication, these proteins are also implicated during movement of viral genomes through cytoplasm, coordinated by PIPO, interacting with the plasmodesmata and helping the transfer of RNA virions from one cell to another (Grangeon et al., 2012; Vijayapalani et al., 2012).

NIb is an RNA-dependent RNA-polymerase; that acts as a replicase in potyviruses. In this study this protein acts synergistically during mixed infections with TMV. It is argued that viral replicases could not synergise other viral infections, since their activity depends on the recognition of specific viral genomes (Mascia and Gallitelli, 2016). However, it has been reported that several replicases are able to induce synergism or support other infections, either by a secondary function of the protein or by the recognition and initiation of replication of the dependent virus (reviewed in Valli et al. 2011). The number of reported examples for these proteins to be synergistic is limited, but it has been reported for the TMV replicase (Ishikawa et al., 1991), NIb from Tobacco etch virus (TEV) and Tobacco vein mottling virus(TVMV) being able to recognise other potyviral sequences, supporting infections by their activity as replicases (Teycheney et al., 2000).These kind of research highlights the importance of thorough risk assessment and evaluation that could be done of transgenic cassava crops expressing intact or partial CBSV viral proteins, highlighting potential interactions that could worsen impacts of other viral diseases in cassava crops.

For the Ham1-like protein, this is the first report where implicates it in potential synergistic interactions, as well as possible antagonistic interactions in the presence of another potyvirus. Its function in the viral genome is still unknown and the ITPase activity reported in Tomlinson et al. (2019a) cannot explain this interaction, however the reduction of PVY titre in plants expressing CBSV Ham1 showed that this gene in CBSV is the only one leading uniform reduction of viral titre through the 26 days of infection. Antagonistic interactions found in transgenic *N. tabacum* expressing P3, 6K2, NIb and Ham1 from CBSV,

might be explained by virus exclusion in infected cells where competition between two closely related viruses reduces infectivity in one or both viruses (Dietrich and Maiss, 2003). There are very few reports of antagonistic interactions between unrelated viruses (Chávez-Calvillo et al., 2016), however rare cases do exist reporting the prevention of replication between unrelated viruses (Bennett, 1953; McKinney, 1941). Although in our study none of the transgenic lines were resistant to PVY infection, there was a reduction of PVY viral titre which could be the result of interference or down regulation of the replication process, even though this need further investigation.

This work builds on the available knowledge regarding gene function in CBSV and highlights the role that some of CBSV proteins may have in synergising or antagonising with other unrelated viruses. This has relevance in the understanding of potential mixed infections and interactions occurring with CBSV and other viral diseases in the field, as it commonly happens with other members of the Potyviridae, which may not occur with TMV or PVY but with viruses related to cassava plants.

Identification of a silencing suppressor protein in CBSV

Viral silencing-suppressors normally counteract the plant host RNA gene silencing mechanism (Zvereva and Pooggin, 2012), which is triggered by the presence of double-stranded RNA generated during viral replication. There are more than 35 viral silencing-suppressor families identified in plant viruses (Ding and Voinnet, 2007). Their silencing suppressor activities interfere with gene silencing mechanisms mainly by targeting various components of the silencing pathway (Burgyán and Havelda, 2011).

It has been suggested that most, if not all plant viruses encode silencing suppressor proteins (Pumplin and Voinnet, 2013). These might enhance viral accumulation by countering the gene silencing mechanism of the host (Brigneti et al., 1998; Du et al., 2011; González-Jara et al., 2004; Pruss et al., 1997; Voinnet et al., 1999; Zvereva and Pooggin, 2012). During this study 10 of the 11 genes encoded in the CBSV genome were assessed for suppression of gene silencing, performing assays of co-infiltration with GFP in the 16c line in the same manner as it has been done for other viral suppressors of gene silencing such as: 2b (Cucumovirus), P0 (Polerovirus),P19 (Tombusvirus), HC-Pro (Potyvirus), P1b (Ipomovirus), P1 (Ipomovirus), AC2 (Begomovirus) (Hamilton et al., 2002; Hui and Shou, 2002; Mbanzibwa et al., 2009; Pfeffer et al., 2002; Voinnet et al., 1999). In this study the P1 protein from CBSV was confirmed to be the only silencing suppressor protein among all coding sequences in the CBSV genome. Presence of conserved regions, the LRRA domain and the putative zinc finger were proposed to play a crucial role during the silencing suppression activity (Mbanzibwa et al., 2009). In this study the LRRA domain was exchanged for LAAA. The loss of silencing-suppression ability in the mutant confirms that this domain has a vital role for the suppression of gene silencing in CBSV. The same substitution in the infectious clone (CBSV_Tanza_P1_LAAA IC) prevented infection of *N. benthamiana*; CBSV could not reach detectable levels during the assessment of the infection either by ELISA or RT-PCR, indicating that the LRRA domain in P1 has an essential role for disease, presumably through its silencing suppression activity, and showing that P1 is the only CBSV protein able to fulfil this role. This supports the hypothesis

that the LRRA domain in CBSV has the same function as the LxKA domain from P1b in CVYV, possibly interacting with the siRNA and interrupting the formation of the RISC complex (Valli et al., 2011, 2008).

Experimental Procedures

Viral source

CBSV strain Nampula, from the region of Nampula, Mozambique (accession number HM346953) was obtained as *Nicotiana benthamiana* infected material from the Food and Environment Research Agency (FERA York-UK). CBSV strain Tanza, collected from a region in Tanzania was obtained from the infectious clone CBSV_Tanza IC (Duff-Farrier et al., 2019). UCBSV strain Kikombe, collected from a region in Tanzania, accession No. KX753356, was obtained from the infectious clone UCBSV “Kikombe” IC (Duff-Farrier et al., 2019).

For synergism studies, *Tobacco mosaic virus* strain U1, (accession No. V01408.1) and *Potato virus Y* (PVY^{O}) strain Ordinary, (accession No. EF026074) were used, both being propagated in *N. tabacum*.

Construction of plasmids

Each encoded gene in CBSV Nampula strain and HC-Pro from PVY Ordinary strain were amplified by PCR, adding start and stop codons ATG and TAA, using primers described in supplemental Table S3. PCRs were performed using Phusion High-Fidelity polymerase (ThermoScientific™). Viral amplicons were cloned into the pJET 1.2 (Themo Scientific™), then sequenced and cloned into pCambia2300_EC, under the 35S promoter and the tNOS terminator regulatory elements.

For the construction of the modified P1 expression vector and P1 from CBSV Tanza expression vector, the entire P1 from CBSV_Tanza_P1_LAAA IC and P1 from CBSV_Tanza IC were excised and cloned into pCambia2300_EC independently.

Homologous recombination in yeast

The P1 region from the CBSV_Tanza IC was mutated through PCR-based site directed mutagenesis and homologous recombination in yeast. The yeast homologous recombination was performed as described in Gietz and Woods, (2002).

The infectious clone was digested using *PshA*I, the homologous recombination in yeast was performed using overlapping fragments created with the following primers; **P1_LRRA_1st_FW** with **P1_LRRA_1st_RV** and **P1_LRRA_2nd_FW** with **P1_LRRA_2nd_RV (**supplemental Table S4) and according to the strategy presented in Supplemental Figure S1.

The mutated infectious clone, CBSV_Tanza_P1_LAAA IC was recovered from *Escherichia coli* and confirmed by PCR (Supplemental Figure S2), utilizing primers for six regions of the infectious clone (Supplemental Table S4). The CBSV_Tanza_P1_LAAA infectious clone was then used as a template for

the cloning of the CBSV P1_LAAA into pCambia2300_EC, creating the expression vector P1_Tanza_LAAA_pCambia2300_EC, which was recovered from *E. coli* and confirmed by restriction digestion (Supplemental Figure S3). The induced mutation for the LAAA was confirmed by sequencing the P1 region (Supplemental Figure S4)

Agrobacterium-mediated transient silencing-suppression assay

Viral constructs in pCambia2300_EC were transformed into *Agrobacterium tumefaciens* LBA4404 through electroporation. The preparation of *A. tumefaciens* for infiltration was performed as described in Johansen and Carrington (2001). A culture of *A. tumefaciens* transformed with pCambia2300_EC containing the sequence of the green fluorescent protein (GFP) was included for each viral construct during silencing suppression assays. Cultures for each viral construct and GFP construct were mixed in a proportion 1:1. Infiltration was performed with 0.2 mL of the suspension at the abaxial surface of the leaves with a syringe with no needle. Assays were performed using wild type *N. benthamiana* and the transgenic 16c line in *N. benthamiana*, which constitutively express the GFP under the 35S promotor of the *Cauliflower mosaic virus* (CaMV). For each assay of silencing suppression activity, six plants were infiltrated. P1 from UCBSV and HC-Pro from PVY were used as positive controls for their known silencing suppressor activity (Gallois and Marinho, 1995; Mbanzibwa et al., 2009). A single agroinfiltration with the GFP construct into the 16c line *N. benthamiana* was used as positive control for the gene silencing of the GFP *in planta*.

GFP imaging

The expression levels of GFP were monitored five days after agroinfiltration into leaves of *N. benthamiana* 16c line, as described in Johansen and Carrington (2001). GFP was visualised using a trans-illuminator UVP VisiBlue VB-26V, fluorescence emission was photographed with a Canon EOS T2i camera, operated with an exposure time of 15 seconds, ISO value of 800 and F value of 8.0. For epi-illumination, pictures were taken with an excitation filter of 425/60 nm and a barrier filter of 480 nm utilized for visualization of GFP in a Leica MZFLIII fluorescence stereomicroscope.

Virus inoculation

N. tabacum plants were inoculated with TMV and PVY from frozen stocks virus infected leaf material. The leaf material (2 g) was ground at room temperature in 5 mL sterile deionised water with a pestle and mortar. Young opened leaves of *N. benthamiana* and *N. tabacum* were dusted with 600 mesh carborundum powder (Fisher Scientific™) at the adaxial surface of the leaf, and freshly ground inoculum was gently rubbed onto these leaves.

For viral interaction assays with CBSV and/or TMV and CBSV and/or PVY, three biological replicas per infection were established in groups of single and mixed infections, inoculations were performed on the same leaf at the same time. Single and mixed infections were monitored for 26 days looking at symptom development and measuring viral titre.

Viral interaction assays

N. tabacum transgenic lines expressing P3, CI, 6k1, 6k2, VPg, NIa, NIb, Ham1 and CP from CBSV were infected with TMV or PVY, using three biological replicas from each independent transgenic line. Infected plants were monitored and sampled for subsequent ELISA analysis at: 0 and 2 DPI from the inoculated leaf; then at 4, 6, 8, 10, 14, 18, 22 and 26 DPI from systemic infection in upper leaves. TMV and PVY titres from infections in transgenic *N. tabacum* were compared to infections in wild type *N. tabacum*.

Transformation of *N. tabacum* plants.

N. tabacum plants were transformed using *A. tumefaciens* LBA4404 to express each individual encoded gene from CBSV. Using the methodology described in Gallois and Marinho (1995) disc leaves were transformed then selected in Murashige & Skoog (MS) media supplemented with kanamycin 100 µg/mL and 6-benzylaminopurin (6-BAP) 1 mg/L for generation of new shoots. Shooting callus pieces were placed in MS media supplemented with kanamycin 100 µg/mL and α-naphthalene acetic acid (NAA) 0.1 mg/L for the generation of roots.

Confirmation of *N. tabacum* transgenic lines

Transgenic lines of *N. tabacum* were maintained in MS media supplemented with kanamycin 100 µg/mL. RT-PCR was carried out using primers in Table S4, in order to verify the presence of transgene mRNA transcripts in all transformed plants. Relative expression of each viral transcript was measured through Q-RT-PCR. Reactions were carried out using RevertAid First-Strand cDNA Synthesis Kit (ThermoScientific™) for the synthesis of cDNA and Maxima® SYBR Green/ROX (Thermo Fisher Scientific) for the quantitative PCR reactions. Primers and conditions for this analysis are listed in supplemental Table S4. Confirmed transgenic lines and relative expression for each transgene are listed in supplemental Table S1. For each transgene used in this study, three independent transgenic lines were used and propagated as technical and biological replica during experimentation. Transgenic expression of the CBSV P1 gene resulted in very low rates of plant growth, which prevented the use of lines expressing P1 during virus-transgene interaction assays (data not shown).

Semi-quantitative viral titre accumulation analysis.

For the detection of TMV and PVY, an enzyme-linked immunosorbent assay (ELISA) was performed using double sandwich antibody (DAS-ELISA) kits for TMV and PVY mono-cocktail (BIOREBA AG), for the detection of CBSV a triple sandwich antibody (TAS-ELISA) kit from DSMZ was used. Leaves were ground in sample extraction buffer with a ratio of 1:20 (wight/volume). ELISAs were performed in a medium-binding 96-wells microtiter plates (Greiner). Colorimetric reactions were measured after two hours of colour development, using a microtiter plate reader (Spectra Max 190 from Molecular Devices) with a filter for a wavelength of 405 nm.

Data analysis and experiment designing.

The absorbances obtained, reading microtiter plates for ELISA, were normalised to the positive control from each ELISA kit. Analyses for quantification and definition of threshold were performed as described by the manufacturer of the relevant ELISA kits. Values of absorbance were transformed to percentages, taking a mix of the positive control reaction from each kit as the 100 percent of the respective measured virus, this adjustment was performed per microtiter plate, to normalize the data to the same consistent measurement each time, since absorbances vary from plate to plate.

At least two transgenic lines were obtained per CBSV gene in this study, from each transgenic line generated in *N. tabacum*, outlined in supplemental Table S1, three clones were then used per line as biological replicas, and two wells with the same sample in the microtiter plate were used as technical replicas, from which average was used to calculate the mean ± standard error, to estimate significant differences. When infections were performed with different versions of CBSV infectious clones or when transgenic lines and wild type *N. tabacum* were inoculated with PVY or TMV a Kruskal-Wallis test was performed to analyse differences in viral titres between treatments , then treatments were analyses in pairs with Dunn *post hoc* test per day. For the estimation of significant differences between single and mixed infections in *N. tabacum* and *N. benthamiana* plants, a Wilcoxon test was performed. For both analyses the statistical significance was taken at $P<0.05$.

Declarations

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Tables

Table 1. Kruskal-Wallis analysis of TMV and PVY infections on transgenic plants expressing various CBSV genes. Data from the Dunn test demonstrates the comparison with the infections in wild type *N. tabacum* plants. In black are presented analysis from TMV infections showing synergy, in red is presented analysis from the PVY infections showing antagonism, asterisks represent significant difference in viral accumulation in transgenic lines in comparison to infections in wild type *N. tabacum*.

Significant differences present in transgenic plants, infected with TMV or PVY along 26 days. Days Post Infection

Transgenic lines	0	2	4	6	8	10	14	18	22	26
Dunn test				.			0.0662			0.0367
P3 1.2	- -	- -	- -	- -	- -	- *	- -	- -	- -	- -
P3 3.4	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
P3 5.1	- -	- -	- -	- -	- -	- -	* -	- -	- -	- **
Dunn test		0.0438				0.0077	0.0184			0.0406
6k2 1.1	- -	- -	- -	- -	- -	- **	* -	- -	- -	- *
6k2 4.1	- -	- -	- -	- -	- -	- *	** *	* -	- -	- -
6k2 9.1	- -	* -	- -	* -	- -	- **	- -	- -	- -	* -
Dunn test										0.0097
CI 1.1	- -	- -	- -	- -	- -	- -	- -	- -	- -	- *
CI 4.1	- -	- -	- -	- -	- -	- -	- *	- -	- -	- **
CI 6.1	- -	- -	- -	- -	- -	- -	- -	- -	- -	- *
Dunn test							0.0433			0.0084
VpG 4.5	- -	- -	- -	- -	- -	- -	* -	- -	- -	- -
VPG 5.1	- -	- -	- -	- -	- -	- -	* -	- -	- -	- **
VPG 5.2	- -	- -	- -	- -	- -	- -	* -	- -	- -	- *
Dunn test						0.0095	0.0274			0.0321
NIA1.2	- -	- -	- -	- -	- -	- **	** -	- -	* *	* -
NIA 2.1	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
NIA 7.1	- -	- -	- -	- -	- -	- -	* -	- -	- *	- **
Dunn test				0.0466					0.0383	
NIb 1.1	- -	- *	- -	- -	- -	- -	- -	- -	- -	- -
NIb 12.1	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
NIb 12.2	- -	- -	* -	** -	- -	- -	- -	- -	- *	- -
Dunn test				0.0276						0.0216
Ham1 1.1	- -	- -	- -	* -	- -	- *	- -	- -	- -	- **
Ham1 4.1	- -	- *	- -	* -	- -	- -	- -	- -	- -	- *
Ham1 4.2	- -	- -	- -	- -	- -	- *	- -	- *	- -	- *
Dunn test	- -	- -	- -	- -	- *	- -	- *	- -	- -	- -
6K1 4.1										
6K1 6.1	- -	- -	- -	- -	- -	- -	- -	- -	- -	- *

Figures

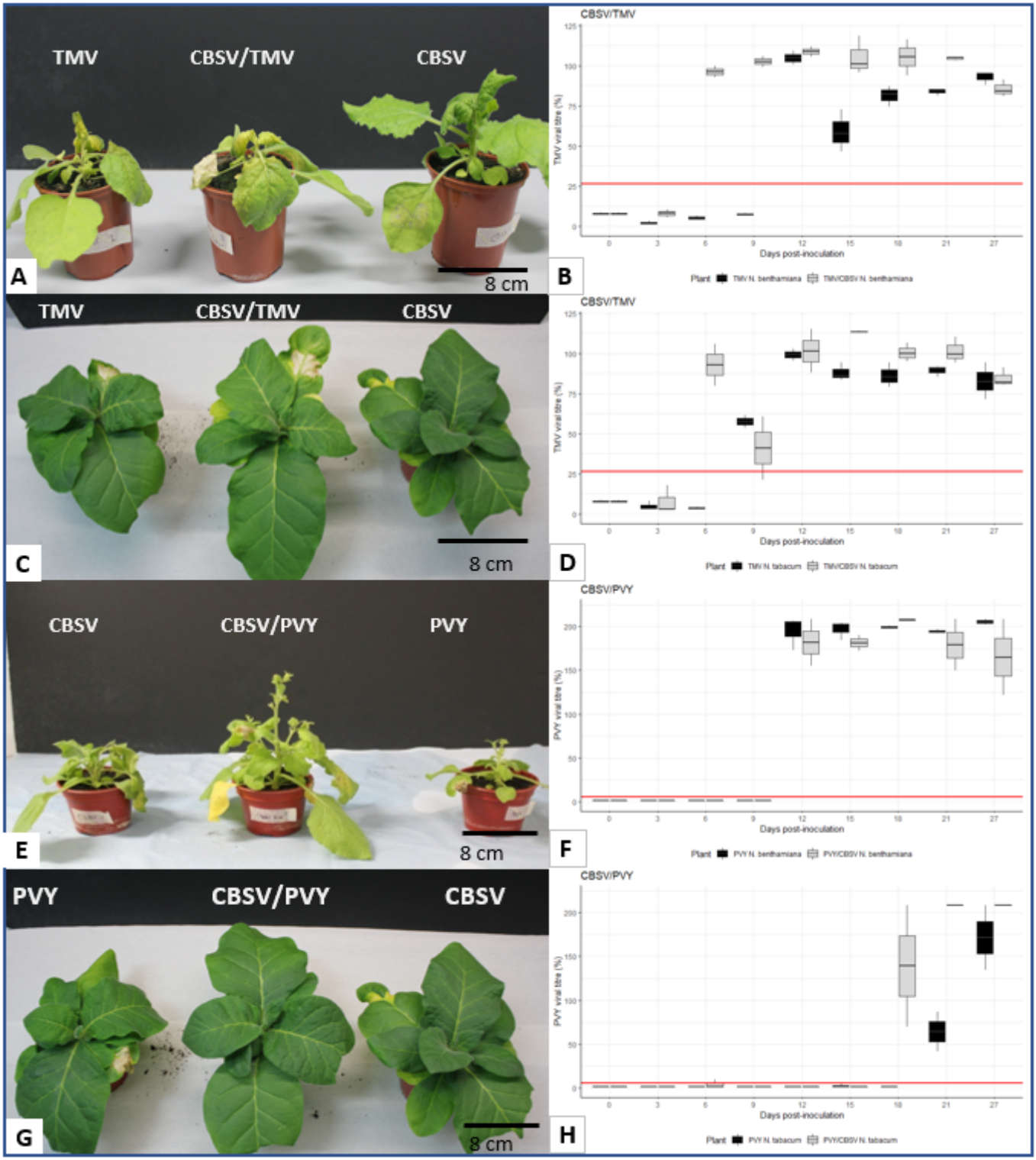

Figure 1

Assessment for synergistic or antagonistic interactions during mixed infections with CBSV/TMV and CBSV/PVY. Left panels show symptoms for single and mixed infections at 15 DPI, which showed synergistic and antagonistic interactions during CBSV/TMV and CBSV/PVY infections, respectively. A-C) *N. benthamiana* and *N. tabacum* plants during CBSV, CBSV/TMV and TMV infections. *N. benthamiana* shows enhanced symptoms for mixed infections with an accelerated necrosis and stunting, whilst *N.*

tabacum mixed infections showed a minimum increase of mosaic and yellowing areas, which TMV or CBSV single infections do not present at this stage. E-G*) N. benthamiana* and *N. tabacum* plants during CBSV, CBSV/PVY and PVY infections. In *N. benthamiana* mixed infections showed a reduction of stunting in comparison to single infections and typical CBSV necrosis was absent during all mixed infection. In *N. tabacum*, mixed infections of these two viruses showed a little reduction in mottling in comparison to PVY single infections.

Right panels show TMV or PVY viral titres measured by DAS-ELISA, when plants were infected with a single virus (black) or combined with CBSV (white). B) Compares TMV titres in *N. benthamiana*, mixed infections show an increase of TMV titre during mixed infections in comparison to TMV single infections. D) Compares TMV titres in *N. tabacum*, mixed infections displayed an early increase in viral titre in comparison to single infections. F) Compares PVY titres in *N. benthamiana*, mixed infections showed a lower viral titter from the 12 DPI up to the 27 DPI in comparison to single infections. H) Compares PVY titres in *N. tabacum*, mixed infection showed similar levels during mixed infections as for single infections.

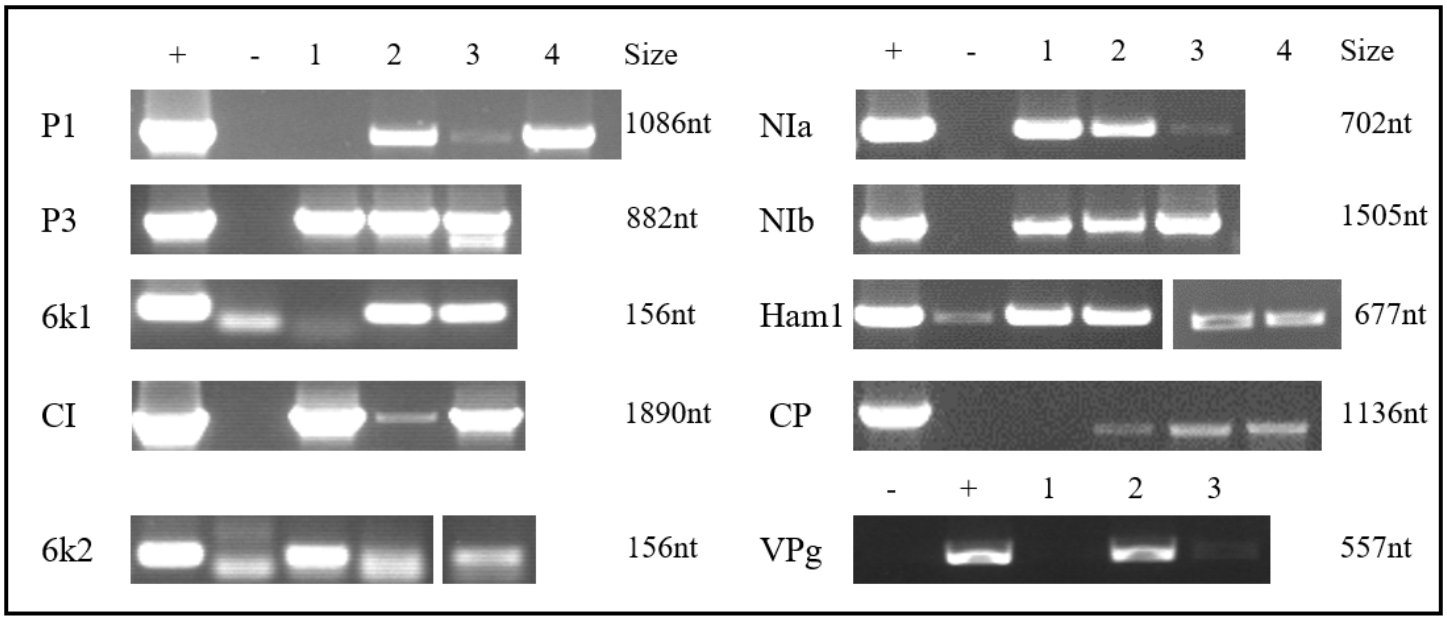

Figure 2

RT-PCR of CBSV genes present in transgenic *N. tabacum* plants, using at least three lines per gene. (+) positive control used for the PCR reaction, (-) negative control used for the PCR reaction, the numbers 1-4 are the number of the *N. tabacum* transformed lines used for the confirmation of CBSV transgenes.

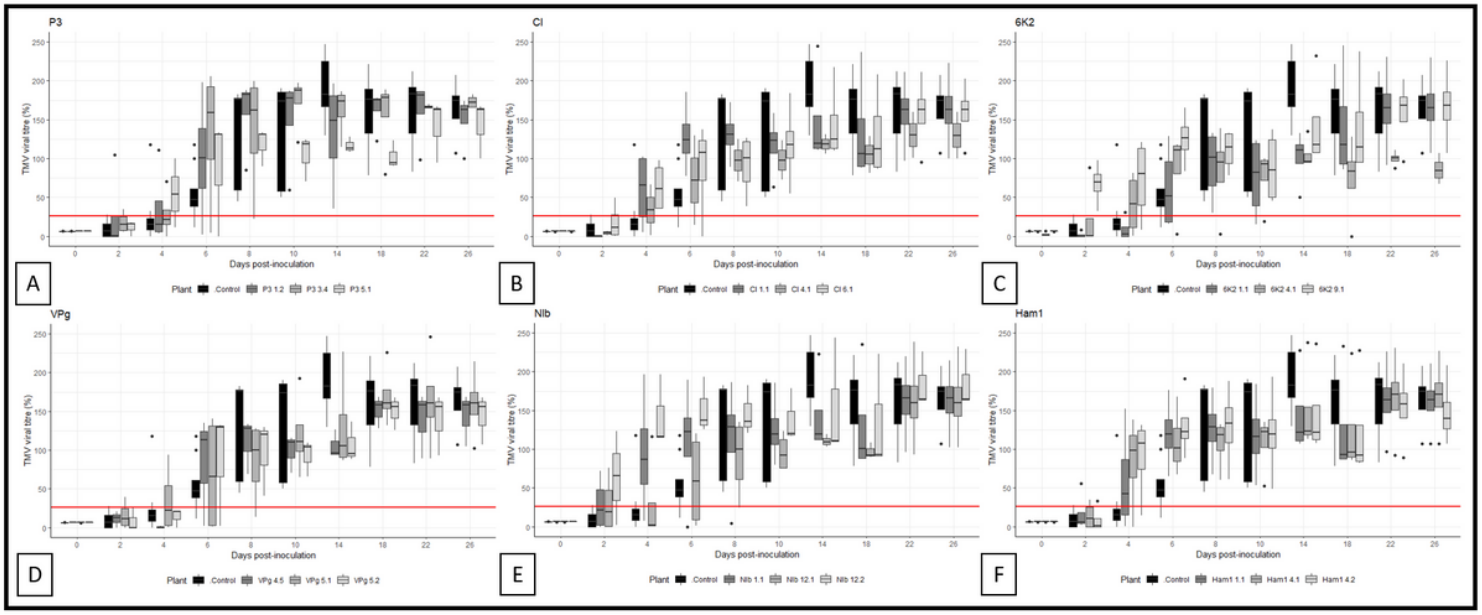

Figure 3

TMV viral titre measures by DAS-ELISA across 26 days of infection in *N. tabacum* transgenic lines expressing genes from CBSV A) lines expressing P3 from CBSV, significant differences are present in line P3-5.1 at 14 DPI, B) lines expressing CI from CBSV, where not significant differences are present but medians tend to be higher than wild type plants at 4 and 6 DPI C) lines expressing 6k2 from CBSV, indicating a significant difference in line 6k2-9.1, showing increased of TMV viral titre at 2, 6 and 26 DPI, also line 6k2-4.1 showed a significant increase at 14 and 18 DPI. D) lines from VPG presented an enhancement at 4 and 6 DPI but later it showed a significant difference by the reduction of TMV viral titre at 14DPI. E) lines expressing Nlb from CBSV, significant differences are present in lines Nlb-1.1 and Nlb-12., showing an increase in TMV viral titre at 4 and 6 DPI. F) lines expressing Ham1-like from CBSV, showed an increase of viral titre from the 2DPI and a significant differences in lines Ham1-1.1 and Ham1-4 at 6 DPI, all in comparison to infections in wild type *N. tabacum* plants.

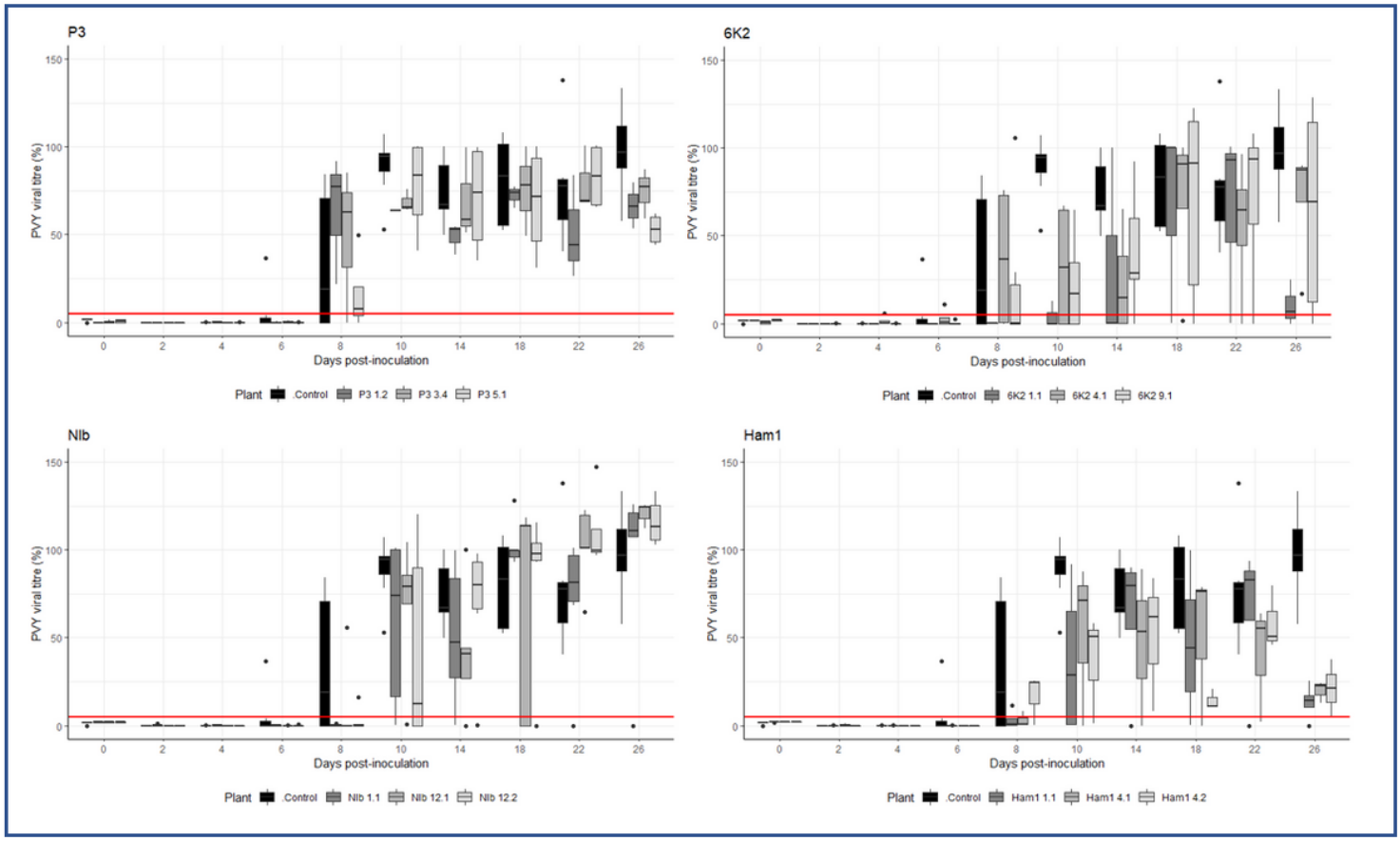

Figure 4

PVY viral titre measured by DAS-ELISA along 26 days of infection with PVY in transgenic *N. tabacum* plants expressing CBSV genes A) lines expressing P3 from CBSV, showed a significant difference at 10 and 26 DPI, B) lines expressing 6k2 from CBSV, displayed a significant difference in titre in all lines at 10 DPI, later at 14 and 26 DPI in 6K2 4.1 and 6K2 1.1 respectively, C) lines expressing Nlb from CBSV showed a significant difference only at 22 DPI but not in a antagonistic manner, D) lines expressing Ham1-like from CBSV showed a trend to reduce viral titre along the 26 days, significant differences were present for the lines Ham1-1.1 and Ham1-4.1, showing reduction of PVY viral titre at 10 DPI and 26 DPI. All are differences are in comparison to the infection in wild type *N. tabacum* plants.

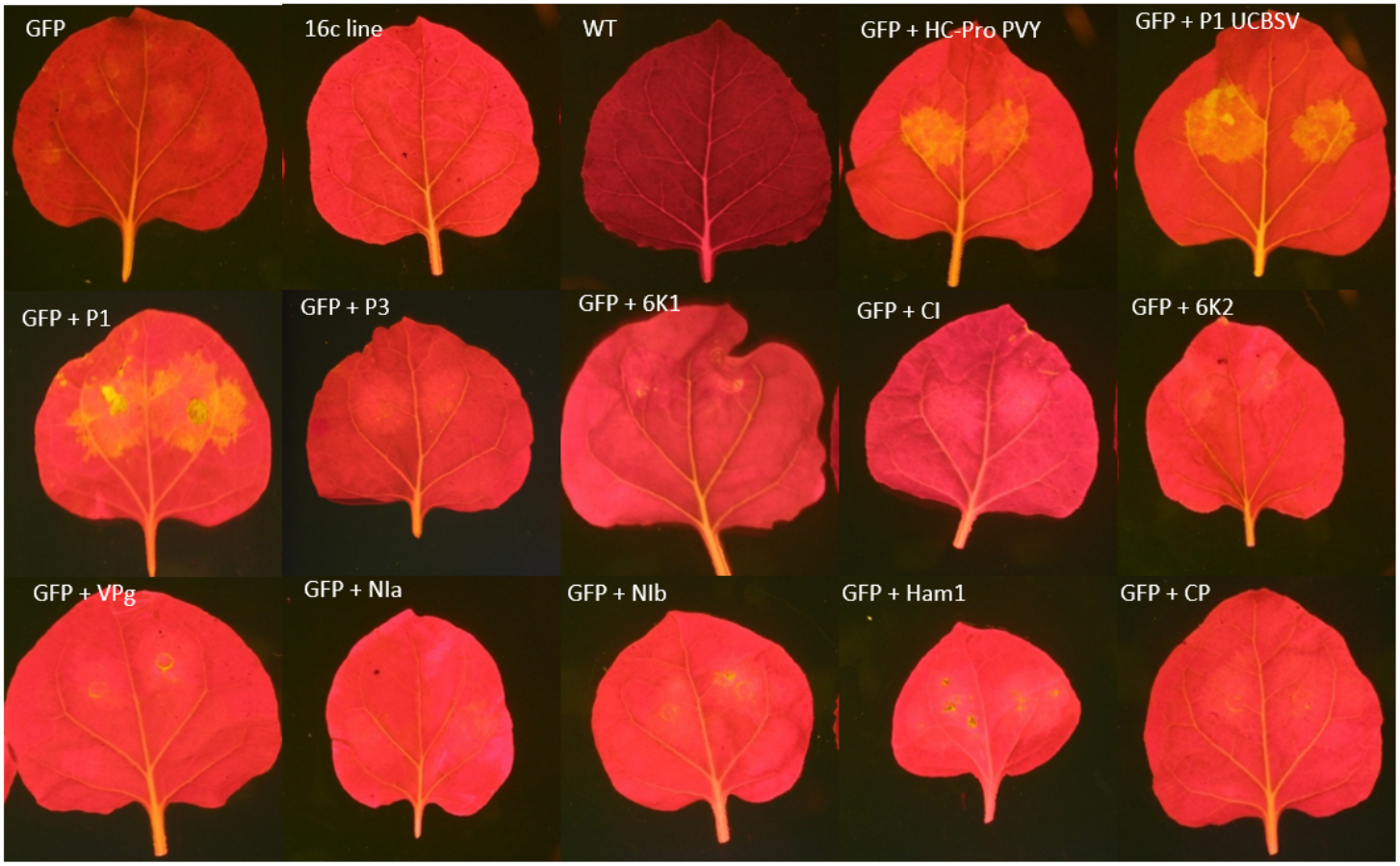

Figure 5

Assays for *in planta* RNA silencing suppression by transient expression of CBSV proteins. Positive silencing suppression is indicated by visible GFP fluorescence. This demonstrates that the P1 protein from CBSV show silencing suppression activity like the positive controls HC-Pro from PVY and P1 from UCBSV. In comparison to the positive controls and P1, the other proteins encoded by CBSV were not able to induce a detectable silencing suppression activity during the agroinfiltration assays. Assays were performed by Agro-infiltration of the 16c line *N. benthamiana* with a GFP expression vector together with expression vectors for: P1, P3, 6K1, CI, 6K2, VPg, NIa, NIb, Ham1 and CP from CBSV. HC-Pro from PVY and P1 from UCBSV were used as positive controls. 16c line infiltrated only with GFP and the wild type (WT) *N. benthamiana* were used as negative controls. Pictures of leaves were taken at 5 DPI. Patches displaying strong expression of GFP (in yellow) indicate a strong silencing suppression activity.

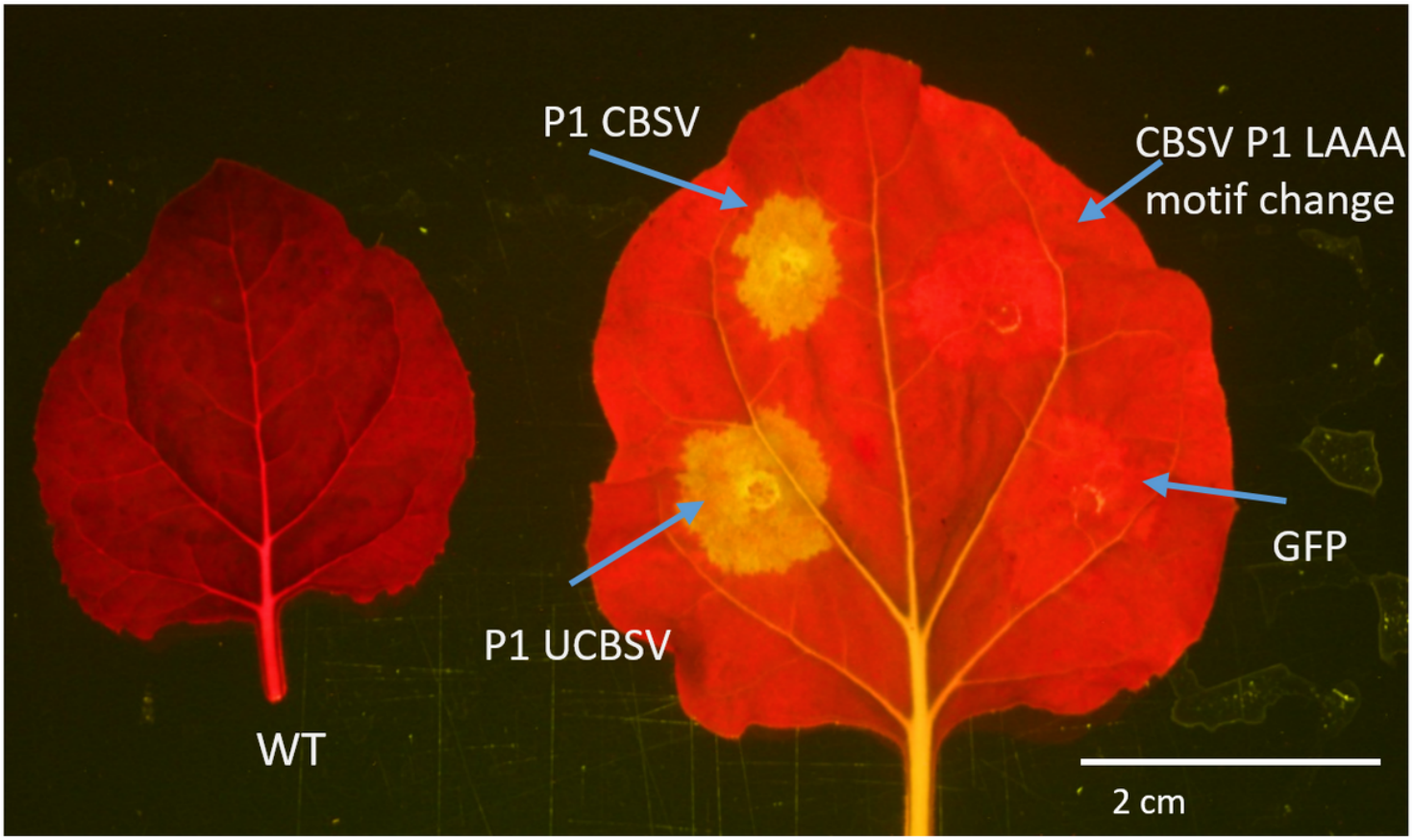

Figure 6

Assessing the silencing suppression ability of the LAAA modified version of CBSV P1. The lack of visible GFP in leaf areas infiltrated with the modified P1 indicate that the sequence change from LRRA to LAAA was sufficient to stop silencing suppression activity. Imaging for the expression of the GFP was taken at 5 DPI, using *N. benthamiana* 16C line, comparing the CBSV P1 LAAA against the non-mutated P1 from CBSV and the positive control P1 from UCSBV Kikombe.

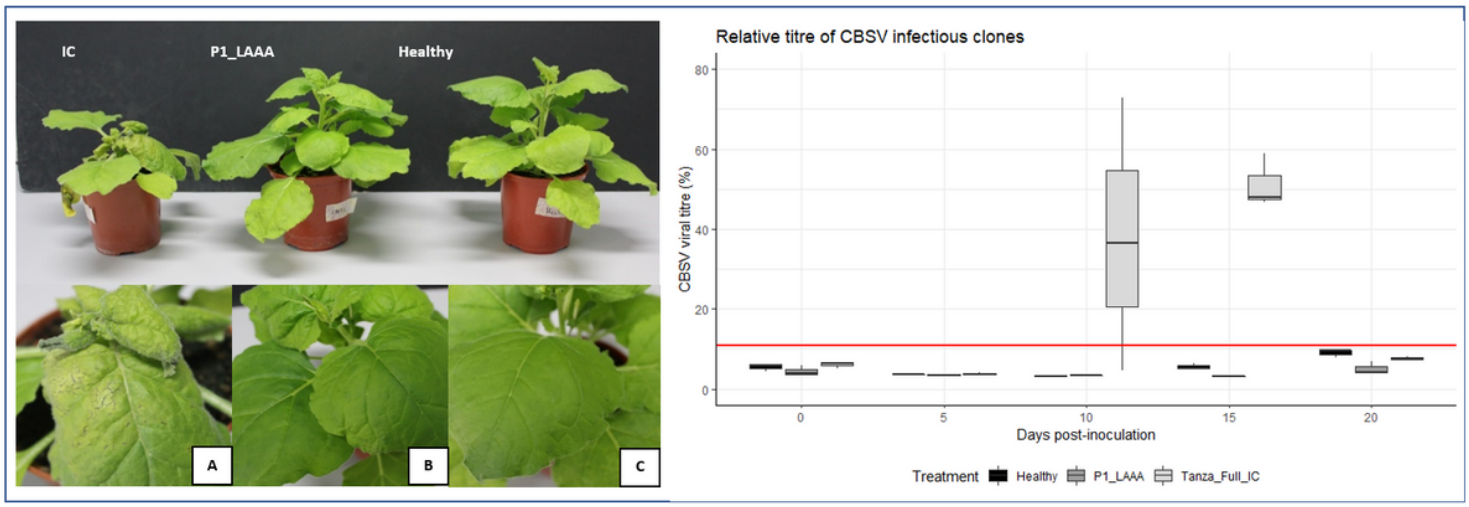

Figure 7

Comparison of infections in *N. benthamiana* plants at 15 DPI inoculated with A) CBSV_Tanza IC "IC", B) CBSV_Tanza_P1_LAAA IC "P1_LAAA" and C) non-inoculated *N. benthamiana* "Healthy". *N. benthamiana* plants inoculated with the P1_LAAA infectious clone did not generate symptoms by the 15 DPI. D) CBSV titre was measured by TAS-ELISA across a 20-day time course for each IC. The P1_LAAA infectious clone did not induce infection at any time point, whilst the wild type infectious clone displayed a high CBSV titre at 10 and 15 DPI. Note that by 20 DPI the entire plant infected with the wild-type clone was severely necrotic, hence the reduced apparent titre.

Supplementary Files

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