Characterization of brown streak virus-resistant cassava

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SUMMARY

Cassava brown streak disease (CBSD) has become a major constraint to cassava production in East and Central Africa. The identification of new sources of CBSD resistance is essential to deploy CBSD mitigation strategies as the disease is progressing westwards to new geographical areas. A stringent infection method based on top cleft grafting combined with precise virus titer quantitation was utilized to screen fourteen cassava cultivars and elite breeding lines. When inoculated with mixed infections of *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), the scions of elite breeding lines KBH 2006/18 and KBH 2006/26 remained symptom-free during a 16-week period of virus graft inoculation, while susceptible varieties displayed typical CBSD infection symptoms at 4 weeks after grafting. The identified CBSD resistance was stable under the co-inoculation of CBSV, UCBSV with cassava geminiviruses (CGMs).

Double grafting experiments revealed that transmission of CBSV and UCBSV to CBSD susceptible top scions was delayed when using intermediate scions of elite breeding lines KBH 2006/18 and KBH 2006/26. Nonetheless, comparison of virus systemic movement using scions from KBH2006/18 and a transgenic CBSD resistant 60444 line (60444-Hp9 line) showed that both CBSV and UCBSV move at undetectable levels through the stems. Further, protoplast-based assays of virus titers over time showed that the replication of CBSVs is inhibited in the resistant line KBH2006/18, suggesting that the identified CBSD resistance is at least partially based on inhibition of virus

replication. Our molecular characterization of CBSD resistance in cassava offers a robust virus–host system to further investigate the molecular determinants of CBSD resistance.

INTRODUCTION

Cassava (*Manihot esculenta,* Crantz) is a food security crop in Africa due to its relatively good performance under difficult growing conditions and the capacity of its starch-rich storage roots to be maintained in the ground, allowing progressive harvest (Fermont *et al.*, 2010). Despite its superior agronomic performance under adverse conditions cassava production is severely constrained by viral diseases. Of these, cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) are the most widespread and severe cassava diseases in Africa (Patil and Fauquet, 2009). While CMD has long been viewed as the main constraint to cassava production in Africa (Seif, 1982; OtimNape *et al.*, 1997), CBSD has recently become a major problem due to its re-emergence in East Africa and its rapid spread into new geographical areas in central African countries (Alicai et al., 2007; Bigirimana et al., 2011; Mulimbi et al., 2012; Patil et al., 2015).

Viral disease management in cassava fields has mostly relied on the identification of existing sources of virus resistance, the introgression of virus resistance traits into farmer-preferred cultivars and the deployment of virus-resistant varieties in the field (Thresh and Cooter, 2005). These strategies have been particularly important for mitigating the impact of CMD in the CMD pandemic regions of Africa (Legg *et al.*, 2006). However, the CMD-resistant cultivars and landraces deployed in CMD-affected regions were not tested for resistance to CBSD. They later appeared to be susceptible to CBSD (Legg *et al.*, 2006), which may have facilitated the spread of CBSD in East and Central Africa during the last two decades. Thus, renewed measures to identify, characterize

and preserve CBSD resistance in cassava germplasm are required for sustainable disease management strategies.

Cassava brown streak virus (CBSV) was confirmed to be the causal agent of CBSD in the 1950s (Lister, 1959), but it was only recently that CBSV was taxonomically grouped into the genus *Ipomovirus* (family *Potyviridae*) (Monger *et al.*, 2001) and that the full genome sequence became available (Mbanzibwa *et al.*, 2009a). Sequencing efforts of CBSD infected cassava samples from different regions in East Africa led to the identification of two viral species (Mbanzibwa et al., 2009b; Monger et al., 2010; Winter et al., 2010; Ndunguru et al., 2015) now referred to as *Cassava brown streak virus* (CBSV), and *Ugandan cassava brown streak virus* (UCBSV). Despite their initial identification from distinct geographical zones, co-occurrence of CBSV and UCBSV has been reported and recent outbreaks of CBSD are not uniquely associated with a particular virus species (Legg *et al.*, 2011; Mbanzibwa *et al.*, 2011a).

CBSD resistance originating from *Manihot glaziovii*, *Manihot melanobasis* and a few cassava varieties of Brazilian origin, was initially reported in the cassava improvement programmes at the Amani Research Station throughout the 1940's and 1950's (Hillocks *et al.*, 2001; Jennings and Iglesias, 2002). However, recent evaluations of the cassava germplasm have identified only tolerance rather than resistance to CBSD (Legg *et al.*, 2011). It remains unclear whether the CBSD resistance was lost through selective breeding for CMD (Hillocks *et al.*, 2001) or that initial reports of CBSD resistance were actually referring to CBSD tolerance due to inconsistent use of the terminology (Cooper and Jones, 1983).

Leaf and root symptoms triggered by CBSD infection vary largely in terms of localization and intensity in susceptible and tolerant cassava varieties (Winter *et al.*, 2010; Mohammed *et al.*, 2012). Molecular tools available today for detecting and discriminating CBSV species offer new opportunities to better determine the tolerance and resistance levels to CBSVs as well as to investigate cassava response to CBSD (Abarshi *et al.*, 2010; Mbanzibwa *et al.*, 2011a; Moreno *et al.*, 2011; Abarshi *et al.*, 2012; Tomlinson *et al.*, 2013). Importantly, such tools should also be used to investigate the robustness of CBSD resistance when exposed to mixed infections of ipomoviruses and cassava mosaic geminivirus (CMG), which do co-occur in several cassava growing regions (Alicai *et al.*, 2007; Legg *et al.*, 2011).

Here we report the screening of selected cassava farmer-preferred varieties and elite breeding lines for CBSD resistance using a robust and reproducible inoculation method in combination with selected CBSV and UCBSV isolates. We monitored viral replication titer over time in order to determine the level of resistance and performed co-inoculation of CBSV and UCBSV isolates with a severe CGM isolate to test the robustness of CBSD resistance under mixed virus infection conditions. We also used double grafting experiments and protoplast-based assays in order to study the resistance mechanism exhibited by the elite breeding line KBH 2006/18 identified as resistant against the CBSV and UCBSV isolates tested in the present study. This first comprehensive characterization of CBSD resistance in cassava under controlled conditions opens new perspectives of investigating the molecular mechanism of CBSD resistance and screening for resistant elite breeding lines and farmer-preferred cassava varieties.

Results

Identification of cassava genotypes resistant to CBSD

Farmer-preferred cassava varieties were selected based on their geographical origin and reported CMD resistance (Supplementary Table 1). Our selection also included elite breeding lines from the Great Lakes Cassava Initiative (CRS, 2012). Disease-free scions were grafted onto cassava variety 60444 rootstocks carrying a mixed infection of CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02]. The top cleft grafting method (Supplementary Figure 1) previously established in our laboratory (Moreno et al., 2011; Vanderschuren et al., 2012) resulted in a high survival rate (over 90%) and 100% CBSD infection in control 60444 scions. CBSD foliar symptoms appeared in scions from susceptible varieties at 4 weeks after grafting (wag) (Table 1). The symptom severity differed between susceptible varieties. In particular, variety MTAI 25 was highly susceptible with early CBSD symptoms followed by dieback of the scions—an observation previously made in the field on highly susceptible cassava varieties (Hillocks *et al.*, 1996; Hillocks *et al.*, 2001). Varieties SC 8, 60444 and TMS 30572 also showed an early onset of CBSD symptoms (Supplementary Figure 2 A) but the scions survived during the 16 weeks observation period. The varieties TME 3, TME 7, UMUCASS 33, KBH 2006/12 and KBH 2002/363 developed mild CBSD symptoms between 4 to 8 wag. The appearance of symptoms on scions of TMS 30001 (a variety that developed few symptoms of restricted distribution when infected with CMGs (Thresh and Cooter, 2005)) was either delayed or not observed in all scions. No CBSD symptoms could be detected in scions of KBH 2006/18 and KBH 2006/26 even at 16 wag (Supplementary Figure 2 B). Results from four independent grafting experiments

are compiled in Table 2. The reported CBSD resistance results in controlled greenhouse conditions are consistent with results from the Great Lakes Cassava Initiative, in which the two elite breeding lines KBH 2006/18 and 2006/26 were identified as CBSD resistant breeding lines in the field (CRS, 2012). However, no further data have been reported on these varieties.

At completion of one grafting experiment, scions from 60444, KBH 2006/18 and KBH 2006/26 were propagated via stem cuttings in the glasshouse. Cuttings of KBH 2006/18 (84%) and KBH 2006/26 (92%) displayed a higher survival rate compared to 60444 (60%) for mixed infection of CBSVs (Supplementary Table 2). All 60444 cuttings showed CBSD symptoms while all propagated KBH 2006/18 and KBH 2006/26 cuttings remained symptom-free (Supplementary Table 2).

We reported earlier a correlation between CBSD symptom severity and virus titers (Moreno *et al.*, 2011). Virus titers were monitored in 60444, KBH 2006/18 and KBH 2006/26 to investigate the presence of CBSVs in symptom-free plants. We quantitated virus levels by reverse transcription-quantitative PCR (RT-qPCR) using the cassava *PP2A* gene as an internal control (Moreno *et al.*, 2011). Quantitation of virus titers in 60444 rootstocks confirmed that scions were exposed to high levels of infection pressure (Supplementary Figure 3). Neither CBSV [TAZ-DES-01] nor UCBSV [TAZ-DES-02] were detectable at 8 wag in the scions of KBH 2006/18 and KBH 2006/26 grafted onto 60444 rootstocks carrying mixed infection of CBSVs (Figure 1 A&B). Both CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] were detected in 60444 scions that showed typical CBSD symptoms. As previously observed (Vanderschuren et al., 2012), UCBSV was on average, detected at higher titers than CBSV in 60444 scions.

Identified CBSD resistance is independent of the grafting procedure

Top cleft grafting on virus-infected rootstocks has proven highly effective for CBSV and UCBSV transmission with 100% infection rates in control plants ((Moreno et al., 2011); Table 2). In order to investigate virus replication in roots, we performed top grafting of CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] infected scions on virus-free rootstocks from a resistant (KBH 2006/18) and susceptible (60444) variety. CBSD symptoms appeared in susceptible 60444 rootstocks in the growing shoots after removal of the inoculating scion at 4 wag. Shoots from KBH 2006/18 rootstocks remained symptom-free. Furthermore, RT-qPCR quantitation of virus titers in storage roots of inoculated rootstocks confirmed the absence of CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] in KBH 2006/18 at 8 weeks after removing the inoculating scion (Figure 2). Propagation of the inoculated rootstock plants via stem cuttings resulted in a low percentage of cutting survival for 60444 compared to KBH 2006/18 (Supplementary Table 3). All established 60444 cuttings developed typical CBSD symptoms while cuttings from KBH 2006/18 remained symptom-free (Supplementary Table 3).

CBSD resistance holds against different CBSV and UCBSV isolates

Resistance of KBH 2006/18 and KBH 2006/26 to CBSD was tested further using UCBSV [UG:Kab4-3:07] – a virus isolate from the epidemic area of Kabanyolo, Uganda (Mbanzibwa *et al.*, 2011b; Mohammed *et al.*, 2012). Top cleft grafting on UCBSV [UG:Kab4-3:07] – infected AR34.2 roostocks was performed using 60444, KBH 2006/18 and KBH 2006/26 scions. UCBSV [UG:Kab4-3:07] titers were quantitated in the rootstock plants (Supplementary Figure 4) and in the scions (Supplementary Figure 5).

High levels of UCBSV [UG:Kab4-3:07] titers could be detected in all susceptible scions but remained undetectable in the scions from KBH 2006/18 and KBH 2006/26 at 8 wag (Supplementary Figure 5).

Additional testing using side grafting method according to Mohammed ~~and colleagues~~*et al.* (2012) was performed to assess resistance of KBH 2006/18 and KBH 2006/26 to mixed CBSV [MZ:Nam1-1:07] and UCBSV [UG:Kab4-3:07] infections. Accumulation of CBSV [MZ:Nam1-1:07] was detected in the susceptible 60444 plants at 8 wag (Supplementary Figure 6). No virus could be detected in the resistant varieties even at 16 wag (Supplementary Figure 6).

KBH 2006/18 is resistant to mixed CBSVs and CMG infection

Co-occurrence of CBSD and CMD has been reported in the field (Thresh *et al.*, 1994; Alicai *et al.*, 2007). However, field data do not support synergism between CBSVs and CMGs (Legg *et al.*, 2011). We inoculated KBH 2006/18 with a single CMG species (ACMV-*NOg* and EACMV-*Ug*, in independent infections) by using the top-grafting method. KBH 2006/18 scions remained symptom-free and control 60444 scions displayed CMD symptoms at 2 wag for both viral species. In the same experiment, we also inoculated scions of the TME 7 landrace that was previously identified as CMD resistant (Fregene *et al.*, 2000; Raji *et al.*, 2008). In contrast to KBH 2006/18 scions that remained symptom-free after CMD infection until the final observation stage at 24 wag, CMD symptoms appeared on the first emerging leaves of TME 7 scions followed by a recovery phenotype typical of CMD tolerant cultivars. Subsequent viral DNA quantitation revealed that ACMV-*NOg* was detectable in both control 60444 and TME 7 scions while

EACMV-Ug viral particles were only detectable in 60444 control scions (Supplementary Figure 7). Both ACMV-*NOg* and EACMV-*Ug* could not be detected in KBH 2006/18 scions.

In order to evaluate resistance of the KBH 2006/18 elite breeding line to a mixed CBSD and CMD infection, disease-free KBH 2006/18 and 60444 scions were grafted on 60444 rootstocks carrying two combinations of CBSVs and CMGs: 1) EACMV-Ug + CBSV [TAZ-DES-01] + UCBSV [TAZ-DES-02], 2) EACMV-Ug + UCBSV [UG:Kab4-3:07]. Viral symptoms appeared in 60444 scions at 3 wag and CMD symptoms were prominent over CBSD symptoms. KBH 2006/18 scions did not show either CMD or CBSD symptoms. Virus detection at 8 wag in inoculated scions revealed that both EACMV-Ug and CBSVs could replicate in 60444 scions (Figure 3). EACMV-Ug and CBSVs titers varied between scions. We also noticed that CBSV [TAZ-DES-01] was not detectable in the 60444 scions grafted on 60444 rootstocks carrying the EACMV-Ug, CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] mixed infection (Figure 3). Viruses used in the mixed infections were below the detection limit in KBH 2006/18 scions. We therefore concluded that CBSD resistance in the elite breeding line KBH 2006/18 holds even when inoculated with mixed infections of CBSVs and EACMV-Ug.

CBSVs are transmitted through KBH 2006/18 scions

In order to test if the elite breeding line KBH 2006/18 restricts viral movement, we performed double-grafting experiments (Supplementary Figure 8 A). KBH 2006/18 and 60444 scions (referred to as first scions) were grafted on susceptible 60444 rootstocks infected with CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02]. Following graft

establishment and stem hardening, uninfected 60444 scions (referred to as second scions) were grafted onto the established first scions. When grafted on 60444 first scions, 60444 second scions developed typical CBSD symptoms as early as 4 wag (Supplementary Figure 8 B). When grafted on KBH 2006/18 first scions, CBSD symptoms in the 60444 second scions only appeared at 10 wag (Supplementary Figure 8 B). CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] were detectable in 60444 first scions but could not be detected in KBH 2006/18 first scions (Supplementary Figure 9). Consistent with symptom development, CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] could be detected in 60444 second scions grafted on 60444 first scions at 4 wag. UCBSV [TAZ-DES-02] could also be detected in 60444 second scions grafted on KBH 2006/18 first scions at 4 wag, even though these second scions were non-symptomatic. Eventually, CBSD symptoms were observed on 60444 second scions from all the grafts at 10 wag (Supplementary Figure 7B). The double grafting experiment clearly indicates that both susceptible and resistant varieties allow movement of CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] through the stem.

In an independent double-grafting experiment, we observed that susceptible second scions grafted onto a CBSD resistant transgenic 60444 (60444-hp9) (Vanderschuren et al., 2012) first scion developed CBSD symptoms at 10 weeks after grafting (Supplementary Figure 8 C). In contrast susceptible second scions grafted onto control 60444-wt scions developed symptoms at 4 weeks after grafting. The similarity in results of double grafting experiments with 60444-hp9 and KBH 2006/18 first scions suggests that those two genetic backgrounds do not differ in restriction of CBSV movement. Our results also suggest that KBH 2006/18 and 60444-Hp 9 have similar resistance levels to

CBSVs since both CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] remained undetectable in the leaves and stems of KBH 2006/18 and 60444-Hp 9 first scions (Supplementary Figure 9).

Virus replication is inhibited in KBH 2006/18 protoplasts

To further elucidate the mode of resistance exhibited by KBH 2006/18, we assayed virus replication in leaf mesophyll protoplasts at 6 hours post-transfection. We extracted virions (Berger and Shiel, 1998) from greenhouse-grown 60444 cassava plants infected with CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] and transfected the virion extract into protoplasts obtained from 60444 and KBH 2006/18 leaves (Yoo *et al.*, 2007). RT-qPCR quantitation of relative virus (CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02]) levels indicated that virus levels increased in 60444 protoplasts over the duration of the experiment. In contrast, UCBSV [TAZ-DES-02] and CBSV [TAZ-DES-01] levels decreased in KBH 2006/18 protoplasts at 6 hours post-transfection (Figure 4). The results of the protoplast replication assay suggest that the characterized CBSD resistance in the KBH 2006/18 elite breeding line is at least partially based on inhibition of one or several step(s) following virus entry in the cell, from uncoating to genome amplification.

Discussion

Natural CBSD resistance is key to control CBSD in African regions where it has become a major constraint to cassava production. This is also critical to minimize the threat of dissemination to regions where CBSD is currently absent. Diagnostics and precise characterization of virus resistance require standard procedures and terminology that

need to be commonly accepted to allow selection and utilization of plant material by virologists and plant breeders. The use of terms for plant responses to virus inoculation has long been debated (Cooper and Jones, 1983). Here we used a stringent top cleft grafting method for inoculation of selected cassava cultivars and elite breeding lines with CBSVs. Our virus inoculation method resulted in 100% infection rates in the susceptible 60444 scions in all experiments. Infection rates obtained by the top grafting method are more consistent and reproducible compared to other inoculation methods reported to date (Maruthi *et al.*, 2005; Mohammed *et al.*, 2012; Ogwok *et al.*, 2012). Because the top grafting method provides a constant virus inoculum from the infected rootstock to the scion, it also allows the assessment of resistance over several weeks of inoculation. Using a mixed CBSV and UCBSV infection we identified two cassava elite breeding lines, KBH 2006/18 and KBH 2006/26, that remained symptom-free even at 16 wag. Cultivar TMS 30001 only developed inconspicuous CBSD symptoms but CBSV accumulated in scions developing disease symptoms.

CBSD infected susceptible varieties usually develop a dry brown-black necrotic rot of the tuberous roots. Despite evidence that CBSVs accumulate in symptomatic and non-symptomatic root tissues (Abarshi *et al.*, 2010; Moreno *et al.*, 2011), the role of root organs in CBSV replication and cycle has not yet been elucidated. Studies in other plant-virus systems suggest that virus accumulation is not homogenous in root systems and that primary roots can sustain high level of virus replication (Dalmay *et al.*, 2000; Valentine *et al.*, 2002). Side grafting and top grafting experiments with CBSV-infected 60444 scions on virus-free KBH 2006/18 rootstocks confirmed that the KBH 2006/18 rootstocks are also resistant to CBSVs. Cumulatively, our data show that the top

grafting method is suitable for identification of CBSD resistance and that resistance against the mixed CBSV – UCSBV infections used in our screen was robust in two elite breeding lines.

CBSV and UCBSV differ in their virulence on cassava cultivars (Winter *et al.*, 2010; Mohammed *et al.*, 2012). We therefore used different combinations of CBSV and UCBSV isolates to assess the stability of the CBSD resistance. KBH 2006/18 and KBH 2006/26 remained symptom-free with all combinations of virus isolates and inoculation methods.

Successful virus disease management of vegetatively propagated crop requires the selection of symptomless cultivars that do not support virus replication and accumulation (van den Bosch *et al.*, 2007). Characterization of plant responses to virus diseases therefore requires molecular quantitation of virus titers in inoculated plants. CBSV and UCBSV were near or below the detection limit in KBH 2006/18 and KBH 2006/26, indicating that these elite breeding lines qualify as resistant (Cooper and Jones, 1983). Comparison of Ct values obtained in RT-qPCR assays of mock controls and inoculated scions of KBH 2006/18 suggests that CBSV and UCBSV do not replicate in leaves of this variety.

Our results also demonstrate that co-inoculation of CBSVs with a severe CMG isolate does not break CBSD resistance in the resistant elite breeding lines. Moreover, co-inoculation does not affect the ratio of the CBSD virus isolates initially present in the susceptible cassava 60444 line, suggesting that both types of viruses do not interfere with their respective replication mechanisms.

Based on the results of our double-grafting experiment, it appears that KBH 2006/18 can transmit CBSVs. The susceptibility of a plant to virus infection depends on both the ability of the virus to gain access to the phloem long-distance transport (Wang *et al.*, 1999; German-Retana *et al.*, 2000) as well as host factors, such as restricted TEV movement (RTM) proteins (Chisholm *et al.*, 2001; Cosson *et al.*, 2010) that permit or inhibit viral movement in vascular tissues. However, the results of double grafting experiments with KBH 2006/18 and a CBSD resistant transgenic 60444 line (60444-hp9) (Vanderschuren et al., 2012) did not reveal any significant difference as both genotypes were able to transmit CBSVs to susceptible second scions, and at similar time points after grafting. Examining virus replication at the cellular level using cassava leaf mesophyll protoplasts also revealed that the CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] titers initially transfected to KBH 2006/18 protoplasts decline over time. On the contraty, both virus isolates had increasing titers over time in 60444 protoplasts. While our protoplast assay results suggest an intra-cellular activity against both CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] isolates, the key virus replication steps, from uncoating to genome amplification, inhibited in the KBH 2006/18 elite breeding lien remained to be identified. The cassava protoplast assay established in the present study will be instrumental to further characterize the presented CBSD resistance. Future characterization of the CBSD resistance in KBH 2006/18 and KBH 2006/26 will also require the development of CBSV and UCBSV infectious clones to determine viral mutations that can overcome the CBSD resistance reported in the present study.

In addition, analysis of cassava varieties with contrasting CBSD resistance using genome sequencing and transcriptome profiling could be particularly instrumental in

identifying genes and their expression patterns that are key for compatible and incompatible interactions in the CBSV – cassava pathosystem. Furthermore, isolation of CBSV isolates capable of breaking CBSD resistance in KBH 2006/18 and KBH 2006/26 elite breeding lines will also provide further insights into CBSV virulence factors.

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Material and methods

Plant material and virus isolates

Disease-free varieties and elite cassava breeding lines used for the study were obtained from international and national research institutes in Africa as well as lines from ongoing breeding programs (Supplementary Table 1). Cassava plants were grown under greenhouse conditions (27°C, 16h light, 60% humidity). The virus isolates were obtained from field infected cassava plants. Mixed infections were generated through grafting and subsequent propagation of scions carrying mixed infections.

Virus inoculation method

Individual plants were assessed for their viral resistance by using the top cleft grafting procedure (Supplementary Figure 1) and side grafting method as previously described (Mohammed *et al.*, 2012). Mock plants consisted of disease-free scions grafted onto disease-free rootstocks. The double grafting procedure involved establishment of the first scion for 14 weeks and subsequent grafting of a second scion following the top cleft grafting procedure. CBSD symptoms were observed at 12 weeks for the plants derived from scions propagated via stem cuttings.

Lower and upper stem samples from 1st scion corresponded to the stem sections 15 – 20 centimeters and 20 – 25 centimeters above the point of grafting, respectively. For detection of CBSVs in the lower and upper stem sections of KBH 2006/18, 60444–Hp 9 and 60444 (Figure 5), the bark of the stems were removed to have a larger fraction of vascular tissues in the samples.

Virus titer quantitation

Total RNA was extracted from leaf samples using a protocol modified from a pine tree RNA extraction protocol (Chang *et al.*, 1993; Moreno *et al.*, 2011). First strand cDNA was synthesized according to the manufacturer instructions (Fermentas) with random hexamer primers mix and 1 µg of total RNA in a final reaction volume of 20 µl. Real-time PCR reactions were performed with the 7500 Fast Real Time PCR System (Applied biosystems, Foster City, CA) using the SDS software. Virus titers were quantitated relative to internal control *MePP2A* as previously described (Moreno et al., 2011). All primers used for virus detection and internal control are listed in Supplementary table 4.

Virion Extraction

CBSV and UCBSV virions were extracted from 20-80g of greenhouse grown, infected leaf material following and adapted potyvirus extraction protocol as described by Berger and Shiel, 1998.

Protoplast isolation and transfection

Cassava leaf mesophyll protoplasts were extracted from mature leaves of *in vitro* grown 60444 and KBH 2006/18 plants and transfected following the Arabidopsis leaf mesophyll protoplast transfection protocol developed by Yoo and colleagues (2007). Total RNA extraction using Isol-RNA (5Prime GmbH) at a ratio of 200 microL of Isol-RNA solution per two million protoplasts was performed. Virus level quantitation was performed as described above. Due to the low amount of RNA obtained in some samples, a cut-off of *MePP2A* Ct <30 was applied to all samples.

Legends

Table 1: CBSD resistance screening on selected cassava varieties and elite breeding lines

Table 2: Summary of all grafting experiments performed

Supplementary Table 1: List of cassava elite breeding lines and varieties used in the study

Supplementary Table 2: Stem propagation of scions inoculated by top grafting on infected rootstocks.

Supplementary Table 3: Stem propagation of rootstocks inoculated by top grafting of infected scions.

Supplementary Table 4: List of primers.

Figure 1: RT-qPCR quantitation of virus titers in scions from selected accessions grafted on rootstocks carrying mixed CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] infection. The Y-axis represents mean fold change of virus *CP* over *MePP2A*. Error bars represent mean ± standard deviation for 3 biological replicates.

Figure 2: RT-qPCR quantitation of virus titers in rootstocks from selected accessions inoculated with scions carrying mixed CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02]. infection. The Y-axis represents fold change of virus *CP* over *MePP2A*. Error bars represent mean ± standard deviation for 3 biological replicates.

Figure 3: Virus detection in KBH 2006/18 and 60444 scions grafted on 60444 rootstocks carrying mixed CBSVs and geminivirus infection at 8 wag. "+" and "-" indicate absence and presence of listed virus isolates in 60444 rootstocks. A. Multiplex PCR with EACMV-AC1 and PP2A primers, B. RT-PCR with CBSDDF2 and CBSDDR primers.

Figure 4: RT-qPCR quantitation of CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] (relative to *MePP2A*) from transfected protoplasts over time. Virus levels are expressed as fold change over control (non-PEG transformed) values to account for only successful transfection events. Error bars represent mean ± standard deviation for a minimum of 3 biological replicates.

Supplementary Figure 1: Schematic representation of the top cleft grafting method

Supplementary Figure 2: Diversity of CBSD symptoms on cassava accessions. A. Leaves from scions inoculated with CBSV [TAZ-DES-01] + UCBSV [TAZ-DES-02]. B. Leaves from scions inoculated with UCBSV [UG:Kab4-3:07]

Supplementary Figure 3: RT-qPCR quantitation of virus titers on 60444 rootstocks carrying mixed infection of CBSV [TAZ-DES-01] and UCBSV [TAZ-DES-02] and used for top grafting of 60444 (A), KBH 2006/18 (B) and KBH2006/26 (C) scions. The Y-axis represents mean fold change of virus *CP* over *MePP2A*. Error bars represent mean ± standard deviation for 3 biological replicates.

Supplementary Figure 4: RT-qPCR quantitation of virus titers on AR34.2 rootstocks carrying UCBSV [UG:Kab4-3:07] and used for top grafting of 60444 (A), KBH 2006/18 (B) and KBH2006/26 (C) scions. The Y-axis represents mean fold change of virus *CP* over *MePP2A*. Error bars represent mean ± standard deviation for 3 biological replicates.

Supplementary Figure 5: RT-qPCR quantitation of virus inoculated plants: UCBSV [UG:Kab4-3:07] detection in scion inoculated by top grafting method on UCBSV [UG:Kab4-3:07] infected rootstocks.

Supplementary Figure 6: CBSV [MZ:Nam1-1:07] detection at 8 and 16 wag in plants inoculated by side grafting method with CBSV [MZ:Nam1-1:07] and UCBSV [UG:Kab4-3:07] infected scions. The Y-axis represents mean fold change of virus *CP* over *MePP2A*. Error bars represent mean ± standard deviation for 3 biological replicates.

Supplementary Figure 7: qPCR quantitation of geminivirus titers in scions inoculated by top grafting method on geminivirus infected rootstocks. The Y-axis represents mean fold change of CMG *AC1* over *MePP2A*. Error bars represent mean ± standard deviation for 3 biological replicates.

Supplementary Figure 8: Summary of the double grafting experiments. (A) The double grafting scheme. Foliar CBSD symptom observation on first and second scions: (B) with KBH 2006/18 first scions and (C) with 60444-hp9 first scions. “+” and “-” indicate the presence and absence of foliar CBSD symptoms for each biological replicate.

Supplementary Figure 9: Detection of CBSV and UCBSV in stems and leaves of KBH 2006/18, 60444-Hp 9 and 60444 scions.

TABLES

Table 1: CBSD resistance screening by top grafting with selected cassava varieties and elite breeding lines

Table 2: Summary of all grafting experiments performed

Supplementary Table 1: List of cassava elite breeding lines and varieties used in the study

Supplementary Table 2: Stem propagation of scions inoculated by top grafting method.

Supplementary Table 3: Stem propagation of rootstocks inoculated by top grafting of infected scions.

Supplementary Table 4: List of primers.

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