CRISPR-Mediated Susceptibility Gene Editing and Viral Interference for Plant Disease Resistance

By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

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Fall 2017

Abstract

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Diseases caused by the family *Potyviridae* require the interaction of viral genome-linked protein (VPg) and host eukaryotic translation initiation factor 4E (eIF4E) isoforms. Disruption of these host genes responsible for *Potyviridae* susceptibility confers disease resistance. CRISPR/Cas9 has emerged as a robust and specific gene editing technology. As a proof of concept for CRISPR-mediated susceptibility gene targeting, *Arabidopsis thaliana* was chosen as a model plant system for editing resistance to *Turnip mosaic potyvirus* (TuMV). CRISPR/Cas9 was employed to generate *eif(iso)4e* mutants in *A. thaliana*. *eif(iso)4e* mutants exhibited resistance to TuMV systemic spread and symptom development. Precise gene editing of *A. thaliana eIF(iso)4E* was also achieved in somatic cells via the CRISPR/Cpf1 tool.

Cassava brown streak disease (CBSD) is a major constraint on cassava yields in East and Central Africa and threatens production in West Africa. CBSD is caused by two species of positive sense RNA viruses belonging to the family *Potyviridae*, genus *Ipomovirus*: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Cassava encodes five eIF4E isoforms: eIF4E, eIF(iso)4E-1, eIF(iso)4E-2, novel cap-binding protein-1 (nCBP-1), and nCBP-2. Yeast two-hybrid analysis detected interactions between both CBSV and UCBSV VPg proteins and cassava nCBP-1 and nCBP-2. CRISPR/Cas9-mediated genome editing was employed to generate *eif4e*, *ncbp-1*, *ncbp-2*, and *ncbp-1/ncbp-2* mutants in cassava cultivar 60444. Challenge with CBSV showed that *ncbp-1/ncbp-2* mutants displayed delayed and attenuated CBSD aerial symptoms, as well as reduced severity and incidence of storage root necrosis. Suppressed disease symptoms were correlated with reduced virus titer in storage roots relative to wild-type controls. However, full resistance to CBSD was not achieved, suggesting that remaining functional eIF4E isoforms may be compensating for the targeted mutagenesis of *nCBP-1* and *nCBP-2*.

Furthermore, viruses of the family *Geminiviridae* threaten to global food security through severe crop loss. *Tomato yellow leaf curl virus* species (TYLCV; genus *Begomovirus*) cause widespread destruction of the tomato crop. The high levels of genetic variability and persistent insect vector distribution pose significant challenges for conventional and genetic control strategies. In addition to precise genome editing, CRISPR/Cas9 may be employed as a form of

molecular immunity against plant DNA viruses. In this study, sgRNA were designed to target a broad range of the TYLCV species, mutagenize a stable region of the TYLCV genome, and disrupt expression of the virus replication machinery. Functionality of multiple TYLCV-targeting CRISPR/Cas9 expression systems was demonstrated in *Nicotiana benthamiana*. The utility of this TYLCV control strategy was then extended to tomato. CRISPR/Cas9 transgenic tomato lines exhibited reduced TYLCV symptom severity and viral DNA presence. CRISPR-mediated viral interference and viral susceptibility gene mutagenesis reflect the vast potential of the CRISPR technology as a solution to global food challenges.

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List of Abbreviations

AUDPC	area under the disease progression curve
BeYDV	*Bean yellow dwarf virus*
BLAST	basic local alignment search tool
bp	base pair
BCTV	*Beet curly top virus*
CaMV	*Cauliflower mosaic virus*
Cas9	CRISPR associated protein 9
CBSD	Cassava brown streak disease
CBSV	*Cassava brown streak virus*
CBSV-Nal	CBSV Naliendele isolate TZ:Nal3-1:07
CLCuKoV	*Cotton leaf curl kokhran virus*
CRISPR	clustered regularly interspaced short palindromic repeats
DSB	double strand break
eIF4E	eukaryotic translation initiation factor 4E
FEC	friable embryogenic calli
GFP	green fluorescent protein
GG	Golden Gate
IITA	International Institute of Tropical Agriculture
INDEL	insertion or deletion mutation
LB	Luria Bertani
MeMV	*Merremia mosaic virus*
MES	2-(N-Morpholino) ethanesulfonic acid
MLO	*mildew-resistance locus O*
MS	Murashige and Skoog
NCBP	National Center for Biotechnology Information
nCBP	novel cap binding protein
VEM	vector-enabled metagenomics
NHEJ	non-homologous end joining
NLS	nuclear localization signal
ORF	open reading frame
PIPO	pretty interesting *Potyviridae* ORF
PlAMV	*Plantago asiatica mosaic virus*
qPCR	quantitative polymerase chain reaction
RESL	restriction enzyme site loss
RNAi	RNA interference
sgRNA	single guide RNA
TuMV	*Turnip mosaic virus*
TYLCD	Tomato yellow leaf curl disease
TYLCV	*Tomato yellow leaf curl virus*
UCBSV	*Ugandan cassava brown streak virus*
UCBSV-T04	UCBV isolate UG:T04-42:04
UV	ultra violet
VPg	viral protein genome-linked
WT	wild-type

Acknowledgments

I would like to acknowledge and thank all the people who supported me during my time as a doctoral student at the University of California, Berkeley. Dr. Brian Staskawicz has been an incredibly supportive advisor through the years. His wisdom and excitement for research have been inspiring from the very beginning of my tenure in the Staskawicz lab. My starting lab mentors, Megan Cohn and Rebecca Bart, were wonderfully welcoming and generous with their time and lab expertise. Lab Manager Doug Dahlbeck was an immense support in keeping the lab running and sharing tips for experiment success. And, I am grateful for my sweet lab neighbor Daniela Thomazella whose bright charm and energy lit up every day at work. And thank you to my qualifying and dissertation committees for pushing me to be an expert in my field and guiding my work to conclusion.

In addition, I would like to acknowledge and thank my collaborators for their contributions to this research. Thank you to Dan Lin, Becky Bart, Nigel Taylor, James Carrington, Getu Beyene, and all other supporting staff at the Donald Danforth Plant Science Center for their expertise in cassava and Cassava Brown Streak Disease. And thank you to Linda Hanley-Bowdoin and Mary Dallas for their partnership in developing tomato yellow leaf curl disease resistance.

Furthermore, I would like to acknowledge and thank my undergraduate research advisor Dr. Imke Schroeder and professor Dr. Asim Dasgupta at the University of California, Los Angeles. Your acceptance, patience, and guidance fostered a curiosity and thrill in studying host-pathogen interactions that keeps me running to this day.

Lastly, I would like to acknowledge and thank my family and friends for their overwhelming support and love. Papi was smiling outside the building the moment I passed my qualifying exam. His warm embrace and frequent visits mean the world to me. Sandrita, mamita, bonita, chiquita, preciosísima, every day you gave me strength and fueled a fire to be a better son. Sean and Dylan, the greatest gifts mom and dad ever gave me were you two. You have been my heroes and partners in crime. And thank you to my dear friends Jeff, Jocelyn, and Michael. I cannot imagine these past years and all their wacky, uplifting, beautiful adventures without you.

From heart to hearts,
Thank you

1. Introduction

Targeted genome editing techniques have emerged as alternatives to classical plant breeding and transgenic methods (Belhaj *et al.*, 2015). The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR associated protein 9) system has rapidly become a favored tool for biotechnology because of its simple design and easy construction of reagents. CRISPR and their associated proteins were discovered in prokaryotes as an adaptive defense strategy against invading viruses and plasmids (Barrangou *et al.*, 2007; Garneau *et al.*, 2010). Archaea and bacteria respond to viral or plasmid challenge by integrating fragments of these foreign sequences into their CRISPR array. From this assembly of repeat and foreign DNA sequences, the CRISPR system produces pre-cursor CRISPR RNA (crRNA) molecules. Following maturation by enzymatic cleavage into short crRNAs, each crRNA assembles into a ribonucleoprotein complex with Cas proteins (Deltcheva *et al.*, 2011; Garneau *et al.*, 2010). Multiple CRISPR/Cas systems have been discovered in prokaryotes, the simplest of which, type II, employs a single Cas9 endonuclease (Chylinski *et al.*, 2014). In the type II system, a trans-activating crRNA (tracrRNA) binds to the crRNA molecule, triggering its maturation and facilitating binding by the Cas9 protein. Guided by the crRNA sequence, this ribonucleoprotein complex performs sequence-specific recognition and cleavage of matching foreign sequences from invading viruses and plasmids. The minimal nature and reprogrammability of the type II CRISPR/Cas system make it an attractive tool for genome engineering applications. Cas9 can be directed to a specific site within the genome via a single guide RNA (sgRNA) (Jinek *et al.*, 2012). Upon binding, Cas9 induces a double strand break (DSB) at the target site (Belhaj *et al.*, 2015). Repair of the DSB by error-prone non-homologous end joining (NHEJ) can generate insertion or deletion (INDEL) mutations that disrupt gene function by altering the reading frame and/or generate a premature stop codon (Britt, 1999; Gorbunova and Levy, 1999).

Resistance to plant pathogens can be controlled either through dominant or recessive gene inheritance. Resistance genes encoding nucleotide-binding leucine-rich repeat receptors, which are dominant sources of extreme resistance against adapted pathogens in many pathosystems, have been cloned and characterized for potyviral diseases, but an overrepresentation in recessive resistance to potyviruses is well documented (de Ronde et al., 2014; Revers and Nicaise, 2014). Recessive resistance to potyviruses is typically associated with mutations in the eukaryotic translation initiation factor 4E (eIF4E) protein family (Bastet *et al.*, 2017; Robaglia and Caranta, 2006). Ethyl methanesulfonate- and transposon-mutagenesis screens in *Arabidopsis thaliana* for decreased susceptibility to *Turnip mosaic potyvirus* (TuMV) identified *eIF(iso)4E* as a loss of susceptibility locus (Duprat *et al.*, 2002; Lellis *et al.*, 2002). More broadly, polymorphisms in eIF4E isoforms of pepper, tomato, lettuce, pea, and other crops confer resistance to numerous potyviruses (Robaglia and Caranta, 2006). The direct physical interaction between potyvirus VPg and specific host eIF4E isoforms is well supported through *in vitro* and *in vivo* binding assays (Kang *et al.*, 2005; Leonard *et al.*, 2000; Schaad *et al.*, 2000; Wittmann *et al.*, 1997; Yeam *et al.*, 2007). In most of these cases, amino acid substitutions within the interaction domains on either VPg or eIF4E isoforms abolished infection. These studies highlight the necessity of eIF4E isoform interaction.

The eIF4E protein family plays an essential role in the initiation of cap-dependent mRNA translation. eIF4E isoforms interact with the 5'-7mGpppN-cap of mRNA and subsequently

recruit a complex of initiation factors for translation. eIF4E and its different isoforms, eIF(iso)4E and novel cap-binding protein (nCBP), vary in degrees of functional redundancy and may have undergone neo- or subfunctionalization (Browning and Bailey-Serres, 2015). Little is known regarding nCBPs, in particular. Studies in *A. thaliana* have shown that nCBP exhibits weak cap-binding, similar to eIF(iso)4E, and increased levels in cap-binding complexes at early stages of cell growth (Kropiwnicka et al., 2015, Bush et al., 2009). However, the specialized function of nCBPs remains unknown. Potyviruses hijack the eIF4E protein family via their VPg for translation initiation, genome stability, and/or viral movement (Figure 1-1) (Contreras-Paredes *et al.*, 2013; Eskelin *et al.*, 2011; Gao *et al.*, 2004; Miras *et al.*, 2017; Zhang *et al.*, 2006). Transgenic approaches leveraging amino acid changes that abolish interaction with VPg or loss of the VPg-associated eIF4E protein have previously been implemented as a form of potyviral disease control (Cui and Wang, 2017; Piron *et al.*, 2010; Wang, 2015). The CRISPR/Cas9 technology has been applied to knockout the VPg-associated eIF4E isoform(s) in *A. thaliana* and cucumber, demonstrating an attractive approach for engineering potyvirus resistance (Chandrasekaran *et al.*, 2016; Pyott *et al.*, 2016).

Alternatively, the CRISPR/Cas9 system may be used to provide direct molecular immunity against DNA viruses in plants. In addition to the aforementioned RNA viruses, plants are encounter DNA viruses of the single-stranded *Geminiviridae* family (Hohn *et al*., 2009). *Tomato yellow leaf curl virus* (TYLCV) and other Begomoviruses of the *Geminiviridae* family cause billions of dollars to be lost annually, threatening food security worldwide (Hasegawa *et al.*, 2016). Multiple approaches have been adopted for disease control including insecticide targeting of insect vectors, resistance breeding, and RNAi transgene expression (Lapidot *et al*., 2014; Vidavsky and Czosnek, 1998; Fuentes *et al*., 2016). These approaches have proven to be costly and challenging. CRISPR/Cas9-mediated viral interference has recently emerged as an attractive alternative approach to disease control. Transgenic expression of Cas9 and sgRNA specific for genomic DNA of TYLCV, *Merremia mosaic virus* (MeMV), *Bean yellow dwarf virus* (BeYDV), and *Cotton leaf curl kokhran virus* (CLCuKoV) in *Nicotiana benthamiana* induced INDEL formation in the viral genomic DNA (Ali *et al.*, 2015; Ali *et al.*, 2016; Baltes *et al*., 2015). Consequently, TYLCV accumulation and symptom development in *N. benthamiana* was suppressed (Ali *et al.*, 2015). Carrying CRISPR-mediated viral interference approach from model plants to afflicted crops carries worldwide benefit for food security.

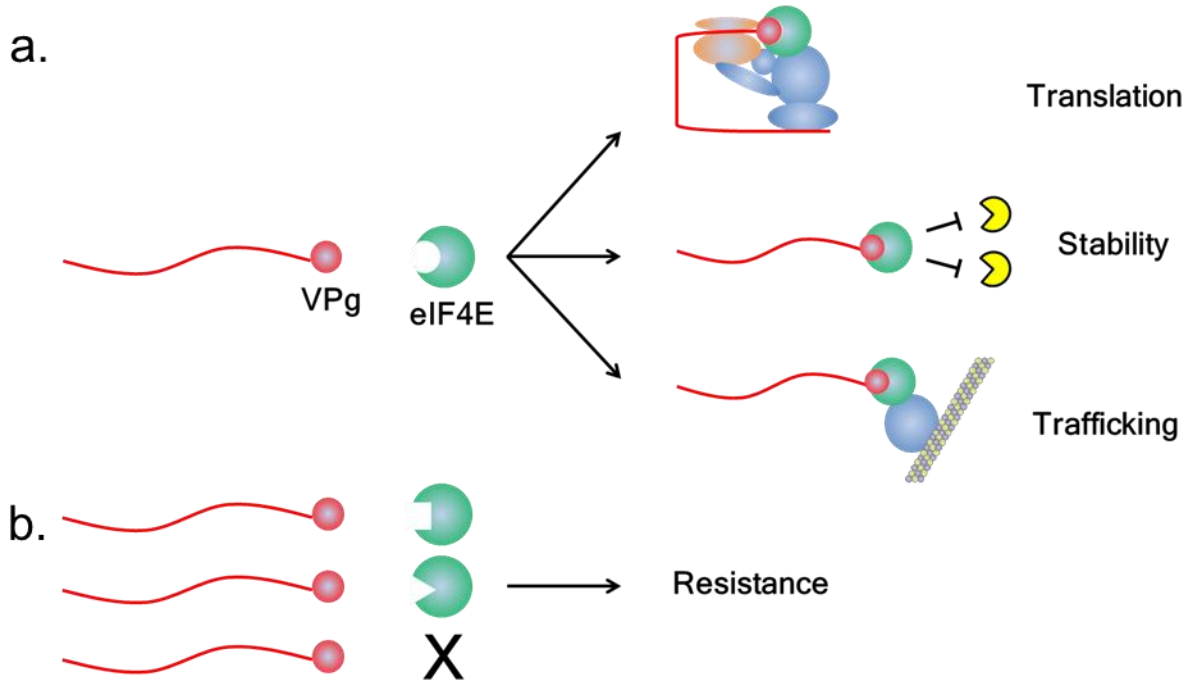

Figure 1-1. Roles of host eIF4E-potyvirus VPg interaction and sources of recessive resistance. (a) Linkage of potyvirus VPg to its binding site on eIF4E can provide translation initiation via recruitment of necessary factors and ribosomal subunits, genomic stability via protection from host-encoded exonucleases, and intracellular trafficking via eIF4G microtubule binding activity. (b) Non-conservative amino acid changes and gene deletions that abolish VPg-eIF4E binding impede above described roles, therefore conferring recessive resistance.

2. CRISPR/Cas9 and CRISPR/Cpf1 mediated editing of potyvirus susceptibility gene *eIF(iso)4E* in *Arabidopsis thaliana*

Background

Sustainable intensification of crop production is critical for achieving long-term food security for a growing human population (Griggs *et al.*, 2013). However, this security is challenged by the *Potyviridae* virus family. *Potyviridae* contains the most abundant group of plant-infecting RNA viruses, which cause significant economic losses in many important crops. *Potyviridae* viruses consist of linear, single-stranded, positive-sense RNA genomes with VPg, a multifunctional protein linked to the 5' end of their genome. Their genomes encode a large polyprotein that is autoproteolytically cleaved to provide mature viral proteins (Adams, 2012). A natural mechanism of resistance to this family is mediated by recessive resistance genes associated with translation initiation factors.

The eukaryotic translation initiation factor 4E (eIF4E) protein family plays an essential role in the initiation of cap-dependent mRNA translation. eIF4E and its isoforms bind to the 5'-cap of mRNA and subsequently recruit a complex of initiation factors for ribosomal translation. In addition, eIF4E and its different isoforms, eIF(iso)4E and novel cap-binding protein (nCBP), are functionally redundant as their respective initiation complexes exhibit complementing activities (Wang and Krishnaswamy, 2012). Potyviruses interact with the eIF4E protein family via their VPg for translation initiation, genome stability, and/or viral movement (Contreras-Paredes *et al.*, 2013; Eskelin *et al.*, 2011; Gao *et al.*, 2004; Miras *et al.*, 2017; Zhang *et al.*, 2006). However, particular amino acid changes or loss of this host factor can provide recessive resistance to potyviruses. Polymorphisms in eIF4E isoforms of pepper (*Capsicum annum, pvr2*), tomato (*Solanum lycopersicum, pot1*), lettuce (*Lactuca sativa, mo1*), pea (*Pisum sativum, sbm1*), and other crops confer resistance to potyviruses (Nicaise *et al*., 2003; Gao *et al*., 2004; Ruffel *et al.*, 2005; Kang *et al*., 2005). Most amino acid changes between potyvirus-susceptible and -resistant genotypes cluster into two surface regions near the cap-binding pocket, though disrupted cap binding is not required for potyvirus resistance (Robaglia and Caranta, 2006).

Within the plant model organism *A. thaliana*, *eIF(iso)4E* has been identified as the gene responsible for *Turnip mosaic potyvirus* TuMV susceptibility via ethyl methane-sulfonate (EMS)- and transposon-mutagenesis screens (Duprat *et al.*, 2002; Lellis *et al.*, 2002). The EMS mutagenesis screen generated mutant lines with single point mutations that resulted in premature stop codons and no phenotypic difference when compared to the wild-type plant. This finding indicates that *eIF(iso)4E* is dispensable for *A. thaliana* growth. Furthermore, the direct physical interaction between potyvirus VPg and specific host eIF4E isoforms is well supported through *in vitro* and *in vivo* binding assays (Kang *et al.*, 2005; Leonard *et al.*, 2000; Schaad *et al.*, 2000; Wittmann *et al.*, 1997; Yeam *et al.*, 2007). Amino acid substitutions within the interaction domains on either VPg or eIF4E isoforms could abolish infection, highlighting the necessity of eIF4E isoform interaction.

A revolutionary tool for genetic engineering has developed over the past several years, namely the CRISPR/Cas9 system. Knockout of the host susceptibility genes via Cas9-mediated

editing should prevent recruitment of the host translation machinery for viral translation and survival. In addition, the CRISPR/Cpf1 system can also be employed for precise genetic engineering. Whereas Cas9 generates a blunt end cut that may be repaired in an unpredictable manner, Cpf1 creates a staggered cut (Zetsche *et al*., 2015). Direct annealing of Cpf1-induced complementary overhangs can yield a predictable repair of the targeted DNA. This study employed CRISPR associated enzymes to edit TuMV resistance in *A. thaliana* by targeting the *eIF(iso)4E* susceptibility gene. At the time of publication, the feasibility of this strategy has been demonstrated in *A. thaliana* and cucumber, bolstering this genetic engineering approach for potyvirus resistance (Chandrasekaran *et al.*, 2016; Pyott *et al.*, 2016).

Results

A. thaliana eIF(iso)4E interacts with TuMV VPg in yeast

Yeast two hybrid analysis was performed to validate the interaction between TuMV VPg and *A. thaliana* eIF(iso)4E. The TuMV VPg protein was fused to the B42 activation domain and transformed into yeast strain EGY48. The *A. thaliana* eIF(iso)4E protein was fused to the LexA DNA-binding domain and transformed into the VPg yeast line. Empty vectors were transformed as negative controls. Five colonies from each transformation were plated on selective media supplemented with X-gal. In this assay, a blue color is indicative of protein-protein interaction dependent activity of the β-galactosidase reporter. *A. thaliana* eIF(iso)4E showed strong interaction with the TuMV VPg (Figure S2-1).

Site-specific mutation of *eIF(iso)4E* by transgenic expression of sgRNA-guided Cas9

To abolish the critical VPg-eIF(iso)4E interaction, CRISPR/Cas9 was employed to generate mutant alleles of *A. thaliana eIF(iso)4E*. Two constructs were assembled to target two sites, positions 18 and 42, within the first exon of *eIF(iso)4E* (Figure 2-1a). Position 18 (pos18) predicted Cas9 cut site overlaps the *Bsl*I restriction enzyme recognition site (Figure 2-1b). Disruption of this recognition site via insertion or deletion (INDEL) formation would result in restriction enzyme site loss (RESL) and immunity to *Bsl*I digestion. Likewise, position 42 (pos42) overlaps the *Mwo*I restriction enzyme recognition site for similar analysis (Figure 2-1c). CRISPR/Cas9 constructs employed the *A. thaliana U6-26* promoter to drive expression of the sgRNA and the Cauliflower mosaic virus (CaMV) 35S promoter to drive expression of human codon optimized *Cas9* fused to sequences encoding an HA tag and two SV40 nuclear localization signals (NLS; Figure S2-2, Figure S2-3). *Agrobacterium* carrying these constructs were then used to transform *A. thaliana* ecotype Col-0 via floral dip (Clough and Bent, 1998). Transgenic T1 plants were selected on growth plates using the *npt*II selectable marker in order to recover plants in which the *CRISPR/Cas9*-containingT-DNA had been integrated into the plant genome. Multiple independent T1 transgenic plant lines were recovered for each construct.

Sites in the *eIF(iso)4E* gene were targeted to disrupt restriction enzyme sequences. Restriction digestion done on PCR products from T1 generation using Bsl1 for pos18 and *Mwo*I for pos42 indicated low-level mutagenesis of the *eIF(iso)4E* gene (Figure 2-2a, Figure 2-3a). Plants that showed the highest level of visible CRISPR/Cas9-induced RESL were grown and

produced seed in isolation. T2 plants were selected on growth plates under kanamycin selection in order to maintain *CRISPR/Cas9* T-DNA expression in hopes of *eIF(iso)4E* gene editing in the germline. T2 plants from pos18 transgenic line showed similar low-level *Bsl*I RESL (Figure 2-2b). Several plant samples from pos42 T2 #4 line showed equal levels of *Mwo*I-digested and *Mwo*I-resistant bands, indicative of higher level RESL and potential heterozygosity between wild-type (WT) and mutant alleles (Figure 2-3b). Restriction enzyme resistant bands from T2 plant samples were sub-cloned into *Escherichia coli* and sequenced via Sanger sequencing. Sequence analysis shows range of insertions and deletions (INDELs) overlapping predicted Cas9 cut site, ranging from single base pair (bp) insertions to 18-bp deletions (Figure 2-4a, b). Selected T2 plants were harvested for seeds which were again plated on selective media for sustained CRISPR/Cas9 T-DNA expression. Homozygous mutants were acquired by the T3 generation consisting of single bp insertions in pos18 line 1-5-1 and pos42 lines 4-4-3 and 4-4-9 (Figure 2-5a, b). These mutations cause reading frame shifts and premature translation stops, likely rendering the protein product non-functional
(Table S2-1).

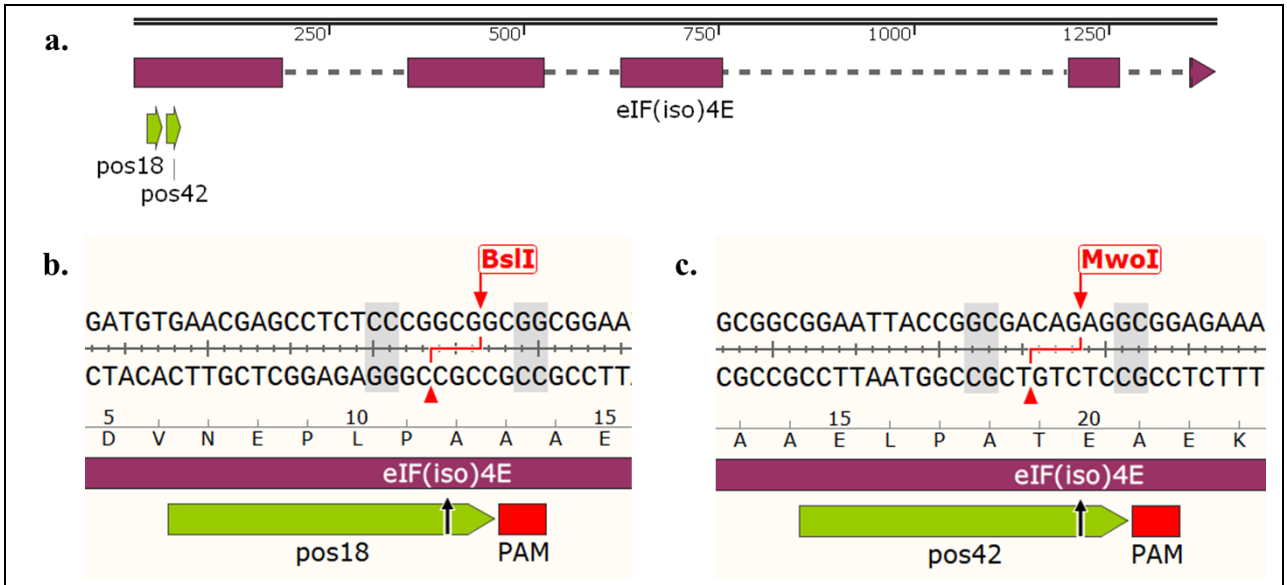

Figure 2-1. *A. thaliana eIF(iso)4E* CRISPR/Cas9 targets overlap restriction enzyme recognition sites. (a) Schematic *of A. thaliana eIF(iso)4E* and Cas9 target positions 18 (pos18) and 42 (pos42). (b), (c) Annotated sequences of A. *thaliana* eIF(iso)4E, pos18, and pos42 features with overlapping restriction enzyme. *eIF(iso)4E* exons are shown in purple, gRNA spacers in green, and protospacer adjacent motifs (PAM) in red. Black arrow represents predicted Cas9 cut site. Red arrows represent restriction enzyme cutting pattern. Gray highlighted sequences represent restriction enzyme recognized sequences.

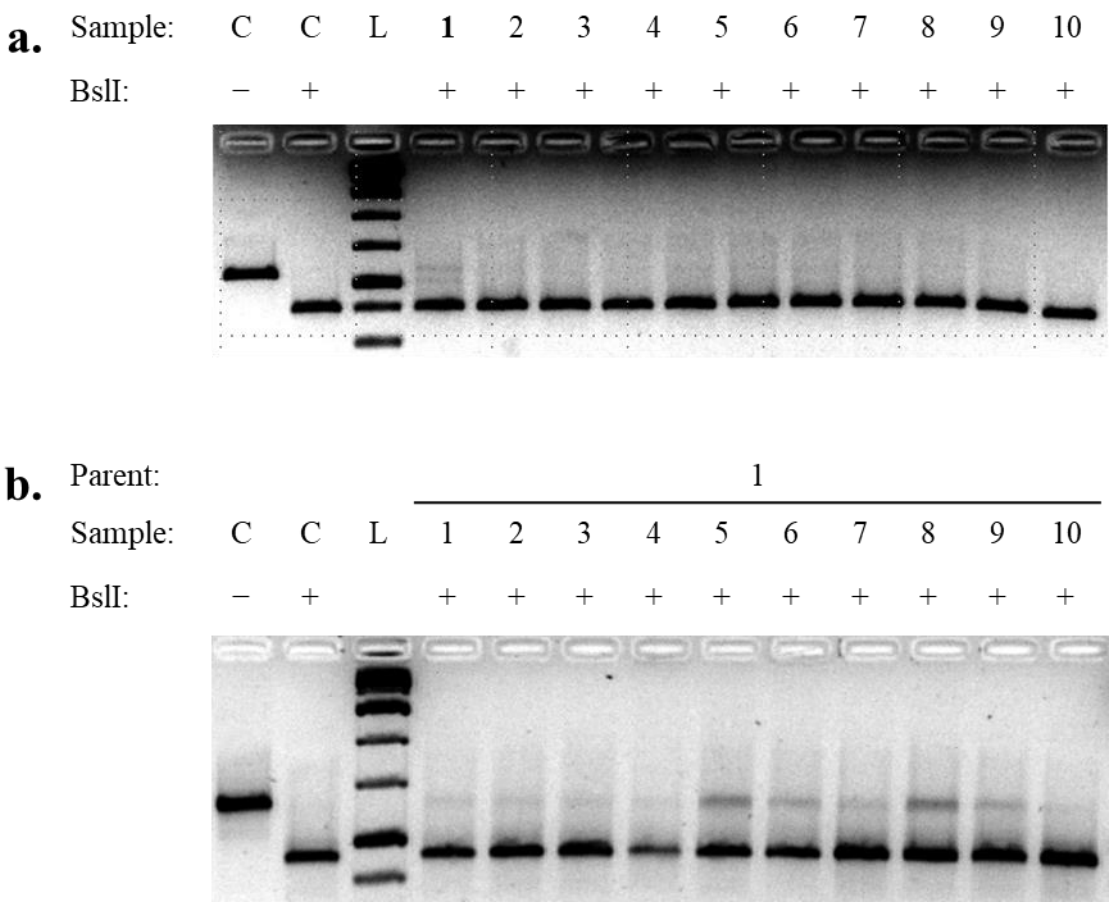

Figure 2-2. CRISPR/Cas9 induces restriction enzyme site loss at *A. thaliana eIF(iso)4E* at position 18 across T1 and T2 generations. PCR amplicons of the Cas9 target region from T1 (a) and T2 (b) transgenic lines were digested by *Mwo*I. Cas9-induced mutagenesis of target site results in RESL. *A. thaliana* wild-type DNA was used for the control, C. DNA ladder, L.

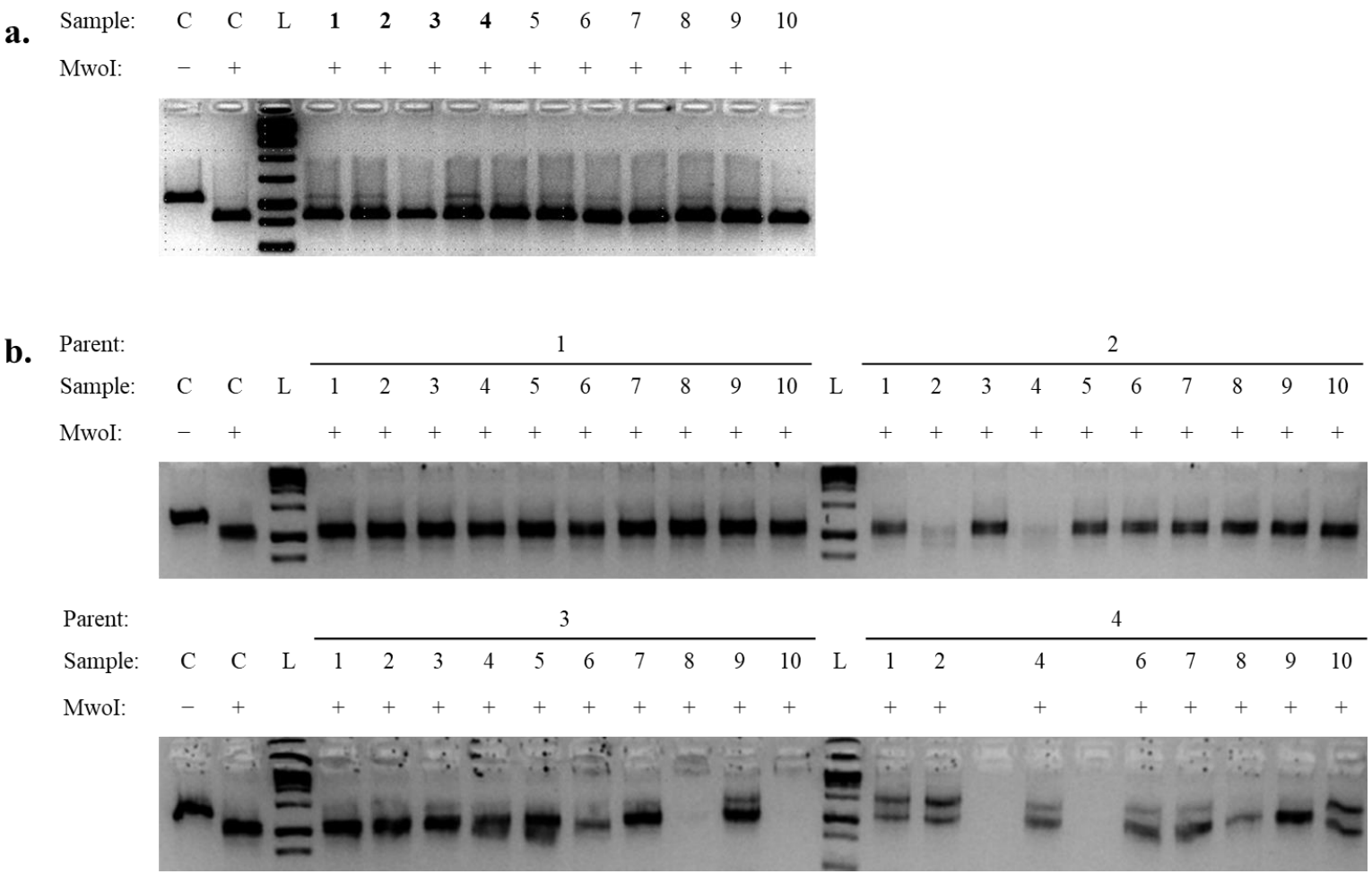

Figure 2-3. CRISPR/Cas9 induces restriction enzyme site loss at *A. thaliana eIF(iso)4E* at position 42 across T1 and T2 generations. PCR amplicons of the Cas9 target region from T1 (a) and T2 (b) transgenic lines were digested by *Mwo*I. Cas9-induced mutagenesis of target site results in restriction enzyme site loss, RESL. *A. thaliana* wild-type DNA was used for the control, C. DNA ladder, L.

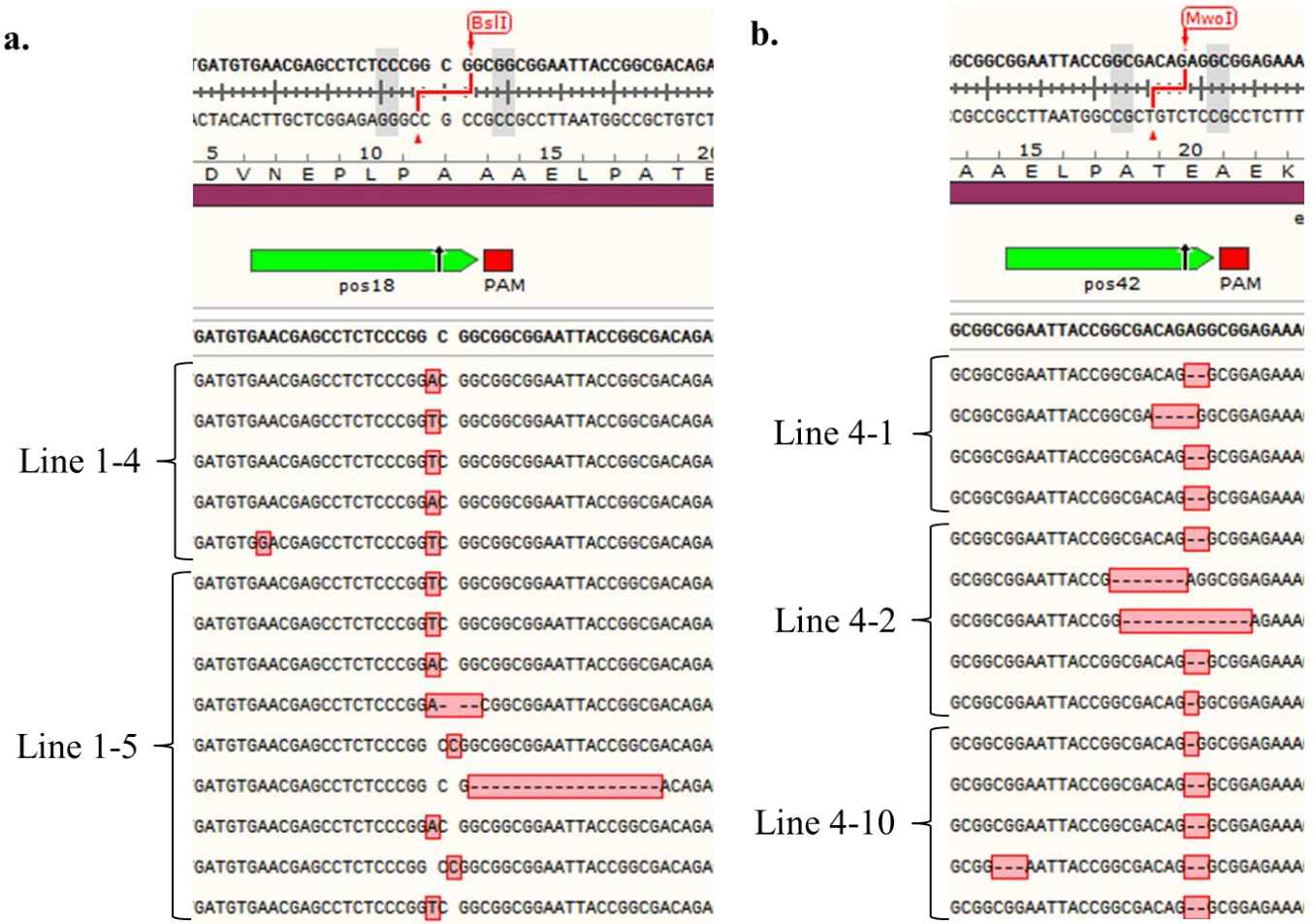

Figure 2-4. Clone sequence analysis of T2 *A. thaliana* shows INDEL formation. Restriction enzyme resistant PCR amplicons were sub-cloned and sequenced. Annotated sequences of *A. thaliana eIF(iso)4E*, pos18, and pos42 features with overlapping restriction enzyme. *eIF(iso)4E* is shown in purple, gRNA spacers in green, and protospacer adjacent motifs (PAM) in red. Black arrow represents predicted Cas9 cut site. Red arrows represent restriction enzyme cutting pattern. Gray highlighted sequences represent restriction enzyme recognized sequences. Red boxed nucleotides represent mismatches to wild-type reference sequence.

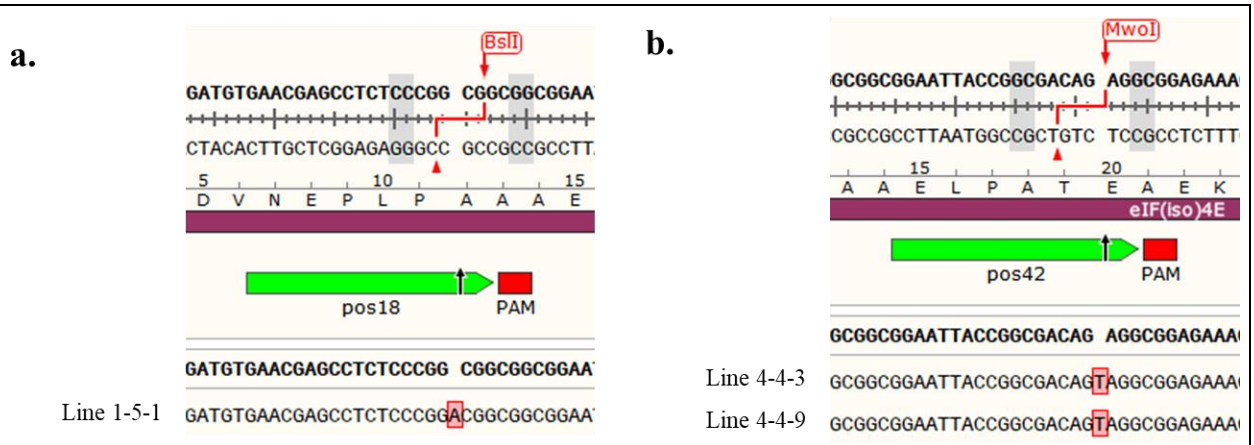

Figure 2-5. CRISPR/Cas9-induced INDELs in *A. thaliana eIF(iso)4E* T3 mutant lines. Annotated sequences of *A. thaliana eIF(iso)4E*, pos18 (a) and pos42 (b) features with overlapping restriction enzymes. *eIF(iso)4E* is shown in purple, gRNA spacers in green, and protospacer adjacent motifs (PAM) in red. Black arrow represents predicted Cas9 cut site. Red arrows represent restriction enzyme cutting pattern. Gray highlighted sequences represent restriction enzyme recognized sequences. Red boxed nucleotides represent mismatches to wild-type reference sequence.

eif(iso)4e mutants exhibit TuMV infection resistance

A green fluorescent protein (GFP)-expressing TuMV was propagated from infectious clone p35-TuMV-GFP (Lellis *et al*., 2002) in *N. benthamiana*. GFP protein was designed for visualizing the accumulation and spread of the virus under UV light. To confirm this virus functionally infected *A. thaliana*, TuMV-GFP leaf extract from *N. benthamiana* was rub-inoculated onto wild-type *A. thaliana* leaves with the abrasive celite. Eight days after infection, systemic spread of TuMV-associated GFP to non-inoculated leaves was visible under ultraviolet (UV) light (Figure S2-4a). Compared to a non-infected control, spread of virus is easily visible on the underside of leaves (Figure S2-4b). A transposon insertion *eIF(iso)4E* mutant that is resistant to TuMV was used as a negative control for subsequent experiments (Duprat *et al.*, 2002). Infections were repeated for three biological replicates of 4-week-old wild-type, transposon mutants, and each mutant line. Leaf underside images of CRISPR/Cas9 *eif(iso)4e* mutant lines taken 15 days after infection show no accumulation and spread of TuMV-GFP (Figure 2-6). Representative positive and negative control leaves were selected for side-by-side image comparison with the CRISPR/Cas9 mutant line leaves.

To ascertain the impact of prolonged infection on *A. thaliana eIF(iso)4E* wild-type and mutant health and morphology, infected plants were grown to 2 months after TuMV-GFP infection. While the infected wild-type replicates experienced severe stunting and stress, the CRISPR/Cas9 and transposon *eIF(iso)4E* mutants exhibited similar growth (Figure 2-7). One transposon mutant replicate perished during transport. Lack of TuMV-associated GFP spread and growth comparable to an established TuMV-resistant mutant indicates the Cas9-induced *eIF(iso)4E* mutant lines are resistant to TuMV infection.

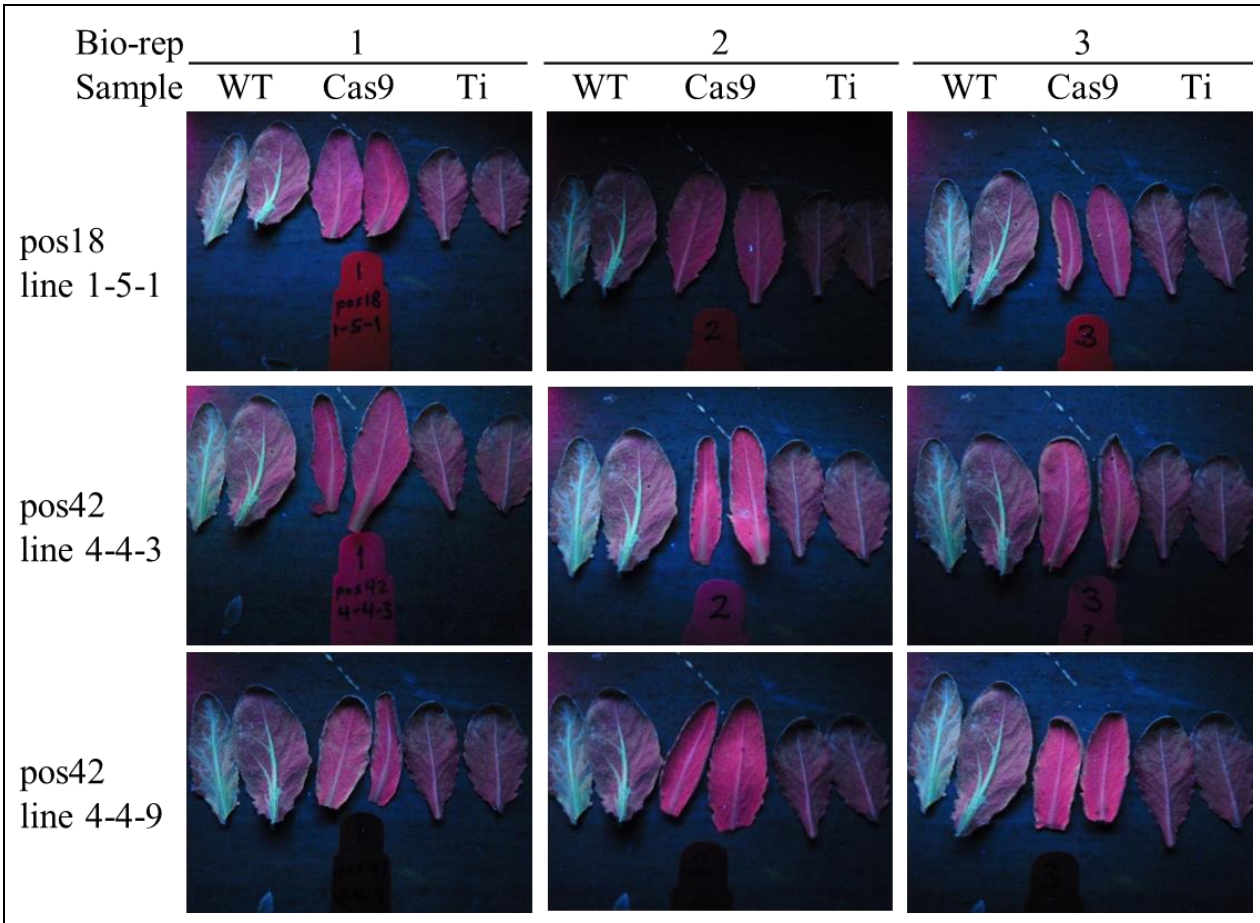

Figure 2-6. CRISPR/Cas9-induced *eIF(iso)4E* mutants show suppressed TuMV-associated GFP expression. Images taken 15 days after infection under UV light. Three Cas9 mutated plants from each line were selected for TuMV-GFP challenge. Fluorescent green regions of leaf reflect systemic spread of TuMV-GFP. Chlorophyll fluoresce red under UV light. Representative wild-type, WT, leaf material was sourced from single plant with vivid TuMV-associated GFP accumulation. Transposon insertion *eIF(iso)4E* mutant (Ti) served as negative control. Two leaves were selected from each plant for photo.

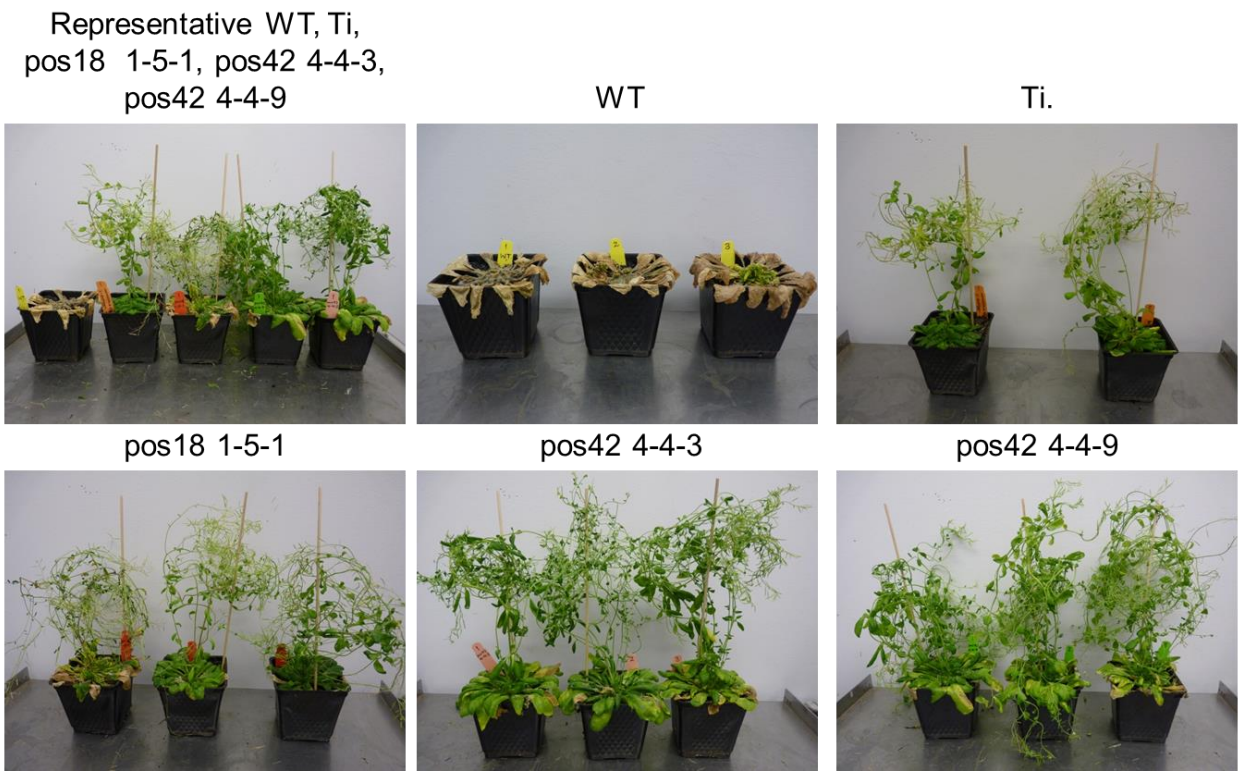

Figure 2-7. CRISPR/Cas9-induced *eIF(iso)4E* mutants show morphology similar to resistant transposon *eIF(iso)4E* mutant, 2 months after TuMV-GFP infection. Top left panel includes representative plants from subsequent panels for side-by-side comparison. Wild-type, WT. Transposon insertion mutant, Ti.

Site-specific mutation of *eIF(iso)4E* by transgenic expression of sgRNA-guided Cpf1

With the emergence of the CRISPR/Cpf1 system, the potential for predictable and precise gene editing was examined. Two Cpf1 target sites were selected encompassing the first exon of *eIF(iso)4E* with predicted complementary overhangs (Figure 2-8). Annealing of the overhangs would excise the whole exon, including the two *Mwo*I restriction enzyme recognition sites. Rather than disrupting the sequence of a restriction enzyme, this approach removes it entirely and is appropriate for RESL analysis. Two CRISPR/Cpf1 constructs bearing the two appropriate sgRNAs were developed using different promoters to drive Cpf1 expression. One construct employs two consecutive CaMV 35S promoters for higher Cpf1 expression (Figure S2-7, Figure S2-8). The other construct employs an egg cell-specific promoter, enhancer, and terminator previously used for effective Cas9-mediated gene editing in the germline by Wang *et al.*, 2015 (Figure S2-9, Figure S2-10). *Agrobacterium* carrying these constructs were then used to transform *A. thaliana* ecotype Col-0 via floral dip.

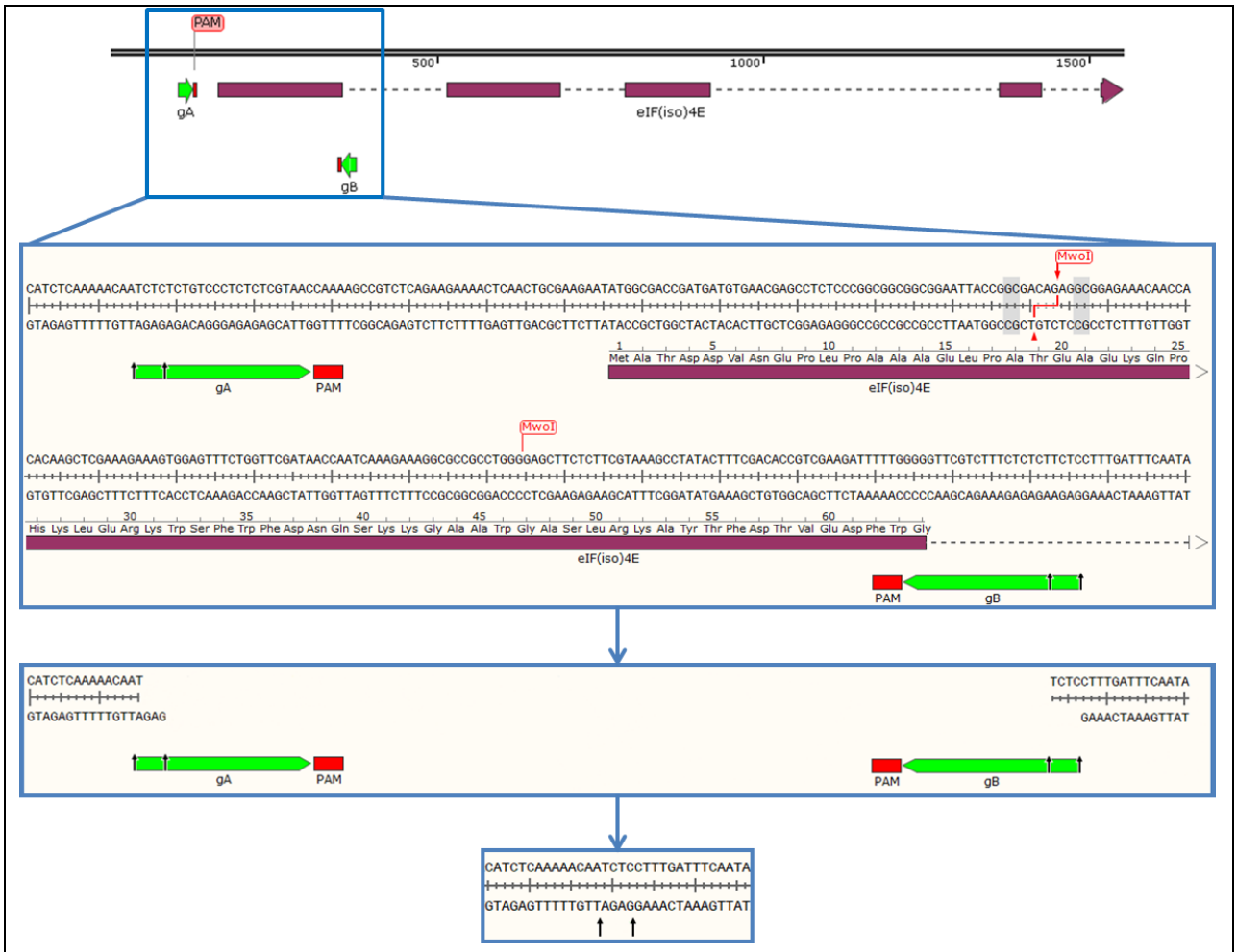

Figure 2-8. Process of predicted CRISPR/Cpf1-induced gene editing of *A. thaliana* *eIF(iso)4E*. Following dual Cpf1 cutting, DNA overhangs are left behind and predicted to anneal to yield a predictable, precise gene edit. Annotated sequences of *A. thaliana eIF(iso)4E*, guide A (gA), and guide B (gB) features with internal *Mwo*I restriction enzyme sites. *eIF(iso)4E* is shown in purple, gRNA spacers in green, and protospacer adjacent motifs (PAM) in red. Black arrow represents predicted Cpf1 cut sites. Red arrows represent restriction enzyme cutting pattern. Gray highlighted sequences represent restriction enzyme recognized sequences.

Transgenic T1 plants were selected on growth plates using the *nptII* selectable marker. Multiple independent T1 transgenic plant lines were recovered for each construct. The presence of the T-DNA backbone and first generation gene editing were examined by PCR amplification of respective fragments. While the CRISPR/Cpf1 T-DNA was present, neither the 2x CaMV 35S- nor EC-driven promoters yielded mutations across both alleles of *eIF(iso)4E* (Figure 2-9a). *Mwo*I-digestion of the genomic DNA followed by PCR amplification showed a band with size consistent with dual Cpf1-mediated exon excision in the 2x 35S plant sample #7 (Figure 2-9b). This band was extracted, sub-cloned, and sequenced to verity Cpf1-mediated mutagenesis. Clone sequence analysis shows a range of INDEL formation including the predicted annealing of the two Cpf1-cut overhangs (Figure 2-10). In all clones, the first exon of *eIF(iso)4E* was excised, consistent with band size and RESL analysis.

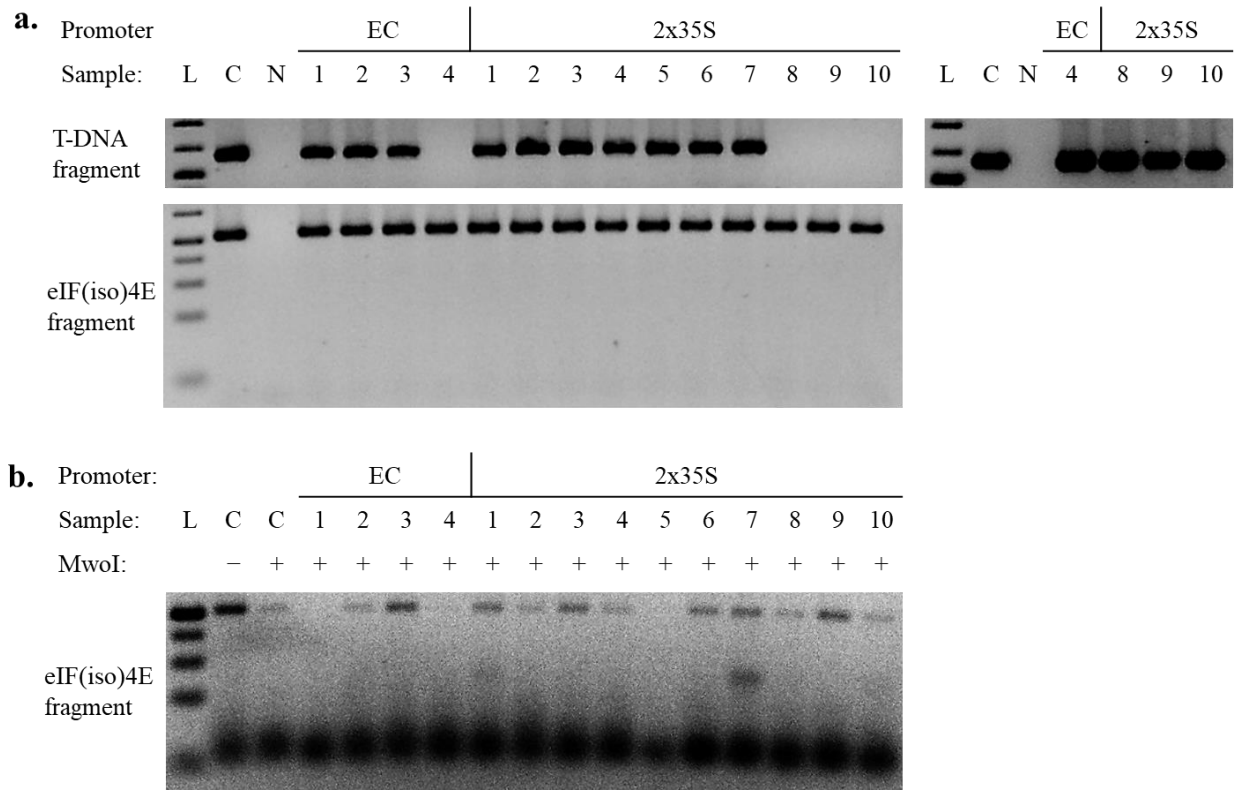

Figure 2-9. CRISPR/Cpf1 induces restriction enzyme site loss at *A. thaliana eIF(iso)4E*. (a) Plant samples were screened for the presence of the Cpf1 T-DNA via PCR. Failed PCR reactions were repeated on far right panel. *eIF(iso)4E* Cpf1 target region was screened for smaller PCR amplicons consistent with dual Cpf1-induced editing of *eIF(iso)4E*. Complete fragment size is 515 bp, and predicted Cpf1-edited fragment is 247 bp. *A. thaliana* wild-type DNA was used for the control, C. No DNA template control, ntc. (b) Dual Cpf1-induced editing of *eIF(iso)4E* results in *Mwo*I restriction enzyme site loss, RESL. 1 µg of genomic DNA from plant samples was digested by *Mwo*I and then the Cpf1 target region was PCR amplified. *A. thaliana* wild-type DNA was used for the control, C. No template negative control, N. DNA ladder, L.

Figure 2-10. CaMV35S-driven CRISPR/Cpf1 plant sample #7 shows predicted Cpf1-mediated gene editing. Reference sequence above shows predicted Cpf1-induced gene edit. Black arrows show predicted Cpf1 cut sites and encompass Cpf1-induced overhangs. Cloned sequences shown below double line. Mismatches between cloned sequences and predicted reference sequence are highlighted in red.

Discussion

In this study, the CRISPR/Cas9 and CRISPR/Cpf1 editing tools were applied for mutagenesis of the *A. thaliana eIF(iso)4E* gene, which is responsible for susceptibility to TuMV. This proof of concept shows the feasibility of rapid crop improvement via CRISPR-mediated gene targeting. Expanding this approach to major crops afflicted by potyviruses carries global benefits for food security. This work is consistent with other efforts to confer resistance to TuMV and other potyviruses via transposon insertion, EMS-, and CRISPR/Cas9-mutagenesis (Duprat *et al.*, 2002; Lellis *et al.*, 2002; Pyott *et al.*, 2016). Together, these studies highlight the necessity of the *A. thaliana* eIF(iso)4E interaction with the TuMV VPg for potyviral infection.

To produce virus *resistant A. thaliana* plants, the CRISPR/Cas9 system was employed to induce precise mutations within the first exon of *eIF(iso)4E*. Targeting overlapping restriction enzyme sites facilitated RESL analysis of the transgenic lines. Future work examining the different Cas9-induced mutations may employ more specific restriction enzymes, the T7 endonuclease assay, or an *in vitro* Cas9 digestion assay. The *Bsl*I and *Mwo*I enzymes used in this study recognized 4 base pairs across a span of 11 base pairs. Therefore, any single or multiple base pair changes that were within the recognition region, were not those four base pairs, and did not shift the reading frame may not have been detected. However, these restriction enzymes are useful for detection of INDELs as these mutations could disrupt the restriction enzyme site. The mutant lines used in our infection assays carried single base pair insertions overlapping the predicted Cas9 cut site. These insertions caused a frameshift that would recognize a pre-mature stop codon. Without a functional eIF(iso)4E, TuMV is unable to recruit this protein for translation, stability, trafficking, and ultimately survival.

This proof of concept is consistent with similar work to engineer transgene-free *A. thaliana* plants that are resistant to potyviruses using CRISPR/Cas9 (Pyott *et al.*, 2016). In their study, the *Petroselinum crispum* polyubiquitin (*PcUbi4-2*) promoter was used to drive

expression of Cas9. A diversity of promoters and promoter arrangements have been employed to optimize expression and boost mutation frequency by Cas9 in *A. thaliana* (Wang *et al*., 2015; Fauser *et al*., 2014; Feng *et al*., 2013; Mao *et al*., 2013). Despite common use of the CaMV 35S promoter for plant gene expression, it may carry weak activity in egg cells and one-cell stage embryos (Wang *et al.*, 2015), thus prolonging efforts to acquire homozygous mutants. The PcUbi4-2 and egg cell promoters described above may be more advantageous for generating homozygous mutants in *A. thaliana* in a single generation. Furthermore, the study by Pyott *et al.*, 2016, carried this approach a step further by segregating away the *eIF(iso)4E* mutation from *CRISPR/Cas9* T-DNA by the third generation. This added step is attractive in the midst of public opinion and government regulations that oppose transgenic foods. By segregating away the *CRISPR/Cas9* T-DNA, the final *A. thaliana* product bears a mutation that could have been engineered via accepted conventional means, such as mutation breeding. This present study was carried out to show the feasibility of engineering CRISPR/Cas9-mediated potyvirus resistance in preparation for application in cassava, *Manihot esculenta* Crantz. Cassava breeding programs for disease resistance are hampered by disruption of desirable trait stacks through sexual recombination, long reproductive cycle, and low fecundity (Ceballos *et al.*, 2004). These challenges make it difficult to segregate away a transgene in cassava, therefore our efforts have focused on engineering the desired mutation. Transgene segregation was not a priority for this proof of concept.

Despite the precise double stranded DNA cutting by Cas9 guided by its sgRNA, the non-homologous end joining pathway to repair the broken DNA is error prone and introduces unpredictable patterns of INDELs (Maruyama *et al.*, 2015). Precise and predictable gene edits may be acquired through homology directed repair in which a repair template is provided. However, this approach had minimal success in *A. thaliana*, likely due to the intrinsically low efficiency of HDR in *A. thaliana* (de Pater *et al*., 2013). An alternative approach is utilization of the CRISPR/Cpf1 tool. Unlike Cas9, Cpf1 induces staggered double strand DNA break (Zetsche *et al.*, 2015). By choosing Cpf1 targets that would yield complementary overhangs, it is possible for those ends to anneal and create a predictable gene edit (Zaidi *et al.*, 2017). This approach was tested by delivery of constructs bearing Cpf1 and gRNA specifically designed to yield complementary overhangs. The predicted gene editing pattern was found through sequencing clones derived from the Cpf1-targeted region of T1 plants. There were other patterns of repair in the form of INDELs; therefore more study is needed to determine the frequency in which the desired gene editing pattern is acquired. Interestingly, no homozygous T1 mutants from utilization of the egg cell promoter were found. It should be noted a very small sample size of these plants were examined. Successful Cpf1-mediated editing using this promoter may be found across a greater sample size of T1 lines. It may also be possible to acquire homozygous Cpf1 mutants under the 35S promoter by progressing to the subsequent generation(s) as was done with the Cas9 transgenic lines.

The CRISPR technology has emerged as an effective tool for inducing precise mutations in plants. In addition to Cas9, Cpf1 expands the molecular toolbox for plant genome engineering, providing a means to engineer desired gene edits. Here, we show the potential for CRISPR tools to engineer potyvirus disease resistance. Expansion of this technology from the *A. thaliana* model to major crops, such as cassava, can have global benefits.

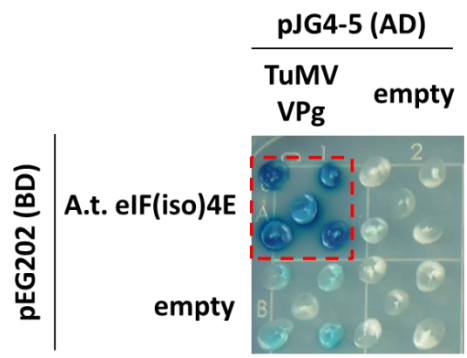

Figure S2-1. TuMV VPg interacts with *A. thaliana* eIF(iso)4E in yeast. Yeast two-hybrid constructs consist of B42 activation domain (AD) fused to the TuMV VPg, and LexA DNA binding domain (BD) bound to *A. thaliana* eIF(iso)4E. Blue coloration represents β-galactosidase activity from activation of lacZ reporter by protein-protein interaction. Five yeast transformants are displayed on the dropout medium SD Gal/Raf SD-UTH.

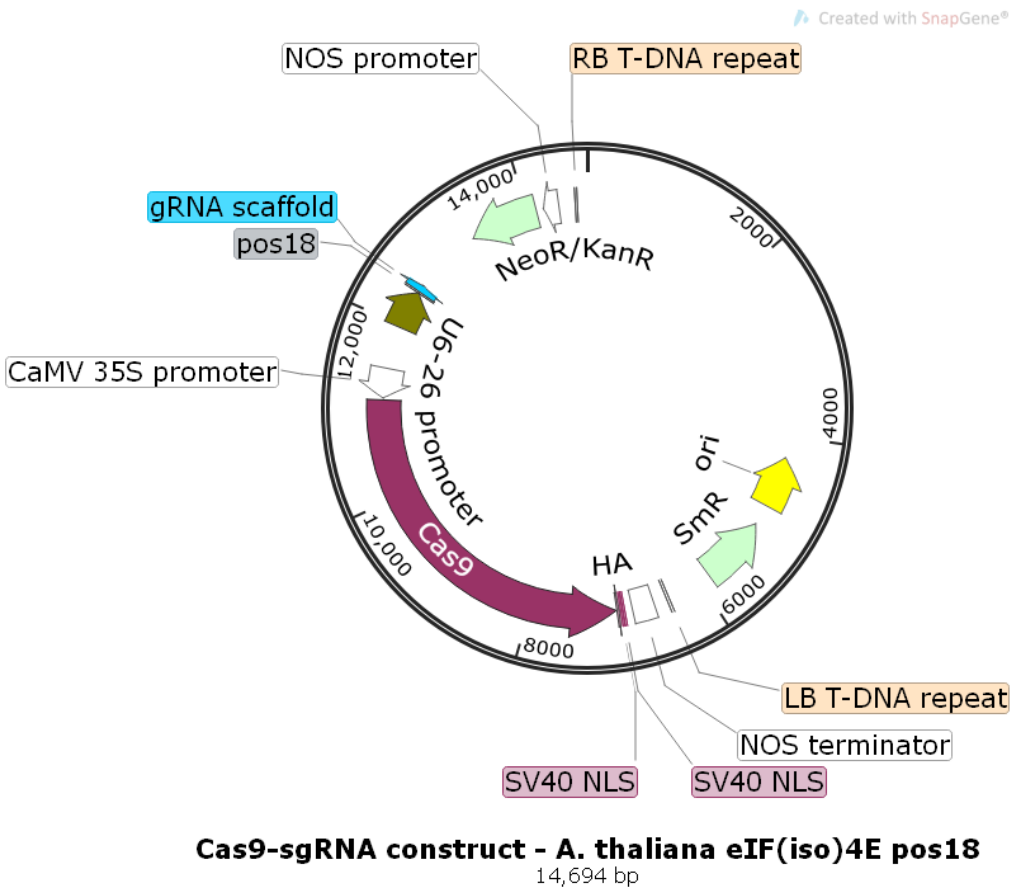

Figure S2-2. Map of CRISPR/Cas9 plant expression vector targeting *A. thaliana eIF(iso)4E* position 18, pos18.

Figure S2-3. Sequence of CRISPR/Cas9 plant expression T-DNA targeting *A. thaliana eIF(iso)4E* position 18, pos18.

TGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTAACG
CCGAATTAAGCTATCAACCACTTTGTACAAGAAAGCTGGGTCGGCGCGATCAACCACTTTGTACAAGAAAGCTGGGTCGGCGCGC
CCACCCTTCCCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTA
TTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAA
CGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTTGA
ACGATCGGGGAAATTCGAGCTCACTCGACTCTAGACTGCAGCAGATCTCAGGGCCCCGGTCGACGTGAGCTCAGGCGCGGCCGCT
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GTACTTAGGCAGCTTGATGATCAGGTCCTTTTTCACTTCTTTGTAGCCCTTGGCTTCCAGAAAGTCGATGGGATTCTTCTCGAAGCT
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AGGAAATCCAGGATTGTCTTGCCGGACTGCTTGTCCCGGATGCCGTTGATCAGCTTCCGGCTCAGCCTGCCCCAGCCGGTGTATCT
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CAGTGTCAGGGTCAGCACGATATCTTCCAGAATGTCCTCGTTTTCCTCATTGTCCAGGAAGTCCTTGTCCTTGATAATTTTCAGCAG
ATCGTGGTATGTGCCCAGGGAGGCGTTGAACCGATCTTCCACGCCGGAGATTTCCACGGAGTCGAAGCACTCGATTTTCTTGAAGT
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AGGCGGGCTTTCTCATTCCCTCGGTCACGTATTTCACTTTGGTCAGCTCGTTGTACACGGTGAAGTACTCGTACAGCAGGCTGTGC
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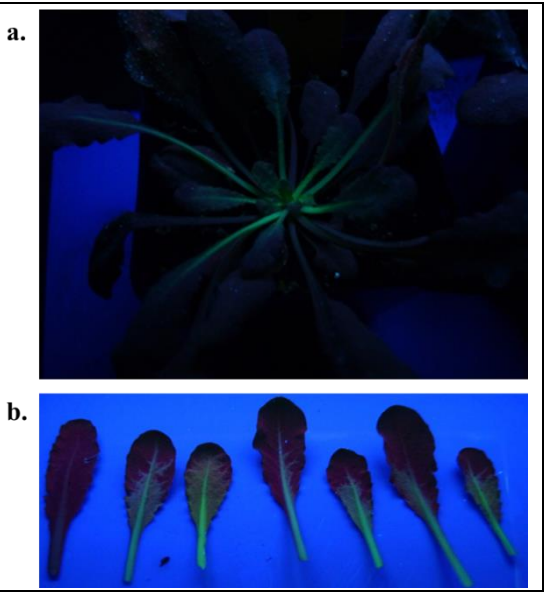
ACTTGTTGATAATTCAGCTTGAGCTTGGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTTGACAGGATATATTGG
CGGGTAAAC

Line	WT	pos18 1-5-1	pos42 4-4-3 pos42 4-4-9
Genotype	WT	A-insertion	T-insertion
Translation	MATDDVNEPLPAAA ELPATEAEKQPHKLE RKWSFWFDNQSKKG AAWGASLRKAYTFD TVEDFWGLHETIFQT SKLTANAEIHLFKAG VEPKWEDPECANGG KWTWVVTANRKEA LDKGWLETLMALIG EQFDEADEICGVVAS VRPQSKQDKLSLWT RTKSNEAVLMGIGKK WKEILDVTDKITFNN HDDSRRSRFTV*	MATDDVNEPLPDGG GITGDRGGETTTQAR KKVEFLVR*	MATDDVNEPLPAAA ELPATVGGETTTQAR KKVEFLVR*

Table S2-1. CRISPR/Cas9-induced mutations in *A. thaliana* cause reading frame shifts and premature translation stops. Wild-type, WT. Stop codon, *.

Figure S2-4. Systemic infection of wild-type *A. thaliana* by TuMV-GFP. (a) Representative photograph of TuMV-GFP infected plants 8 days post infection under UV light. (b) Non-infected, left-most leaf is negative control. Systemically infected leaves from inoculated plant show accumulation of TuMV-associated GFP expression.



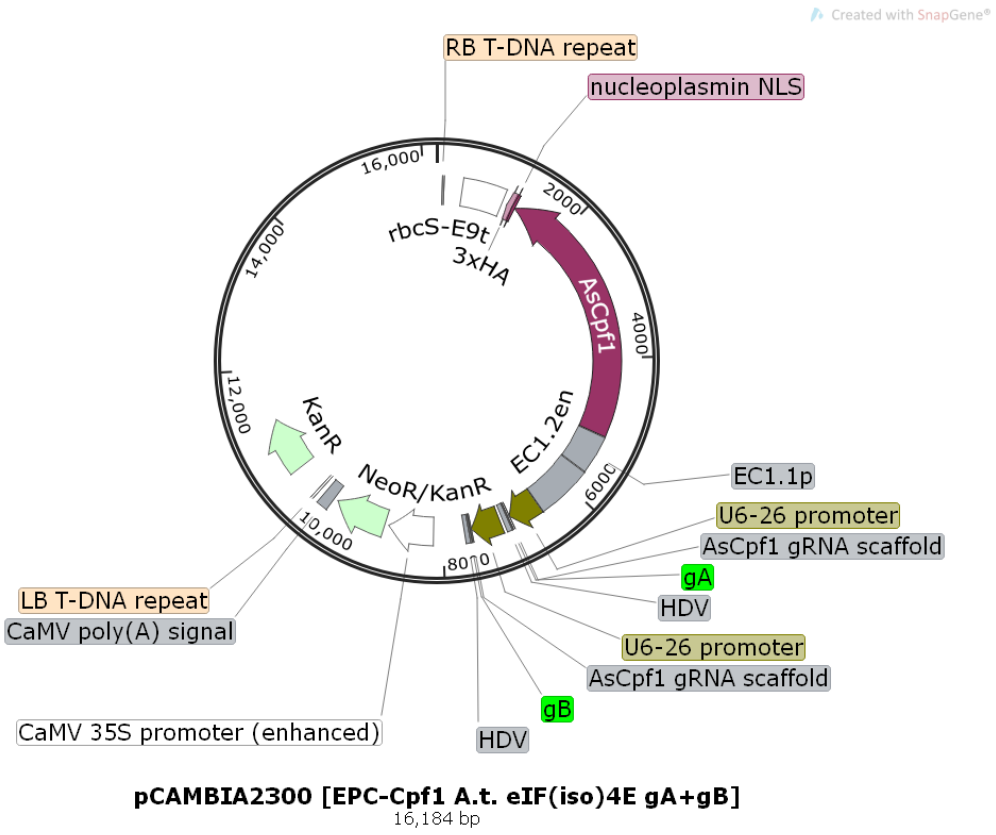


Figure S2-5. Map of *CRISPR/Cpf1* plant expression vector with egg cell promoter.

Figure S2-6. Sequence of *CRISPR/Cpf1* plant expression T-DNA with egg cell promoter.

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TTTCATACTCAACTACAAATCCATGAGTATAACTATAATTATAAAGCAATGATTAGAATCTGACAAGGATTCTGGAAAATTACATA
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ACATTTTTACTCAAAACAAAATGTTTGCATATCTCTTATAATTTCAAATTCAACACACAACAAATAAGAGAAAAAACAAATAATA
TTAATTTGAGAATGAACAAAAGGACCATATCATTCATTAACTCTTCTCCATCCATTTCCATTTCACAGTTCGATAGCGAAAACCGA
ATAAAAAACACAGTAAATTACAAGCACAACAAATGGTACAAGAAAAACAGTTTTCCCAATGCCATAATACTCAAACTCAGTAGG
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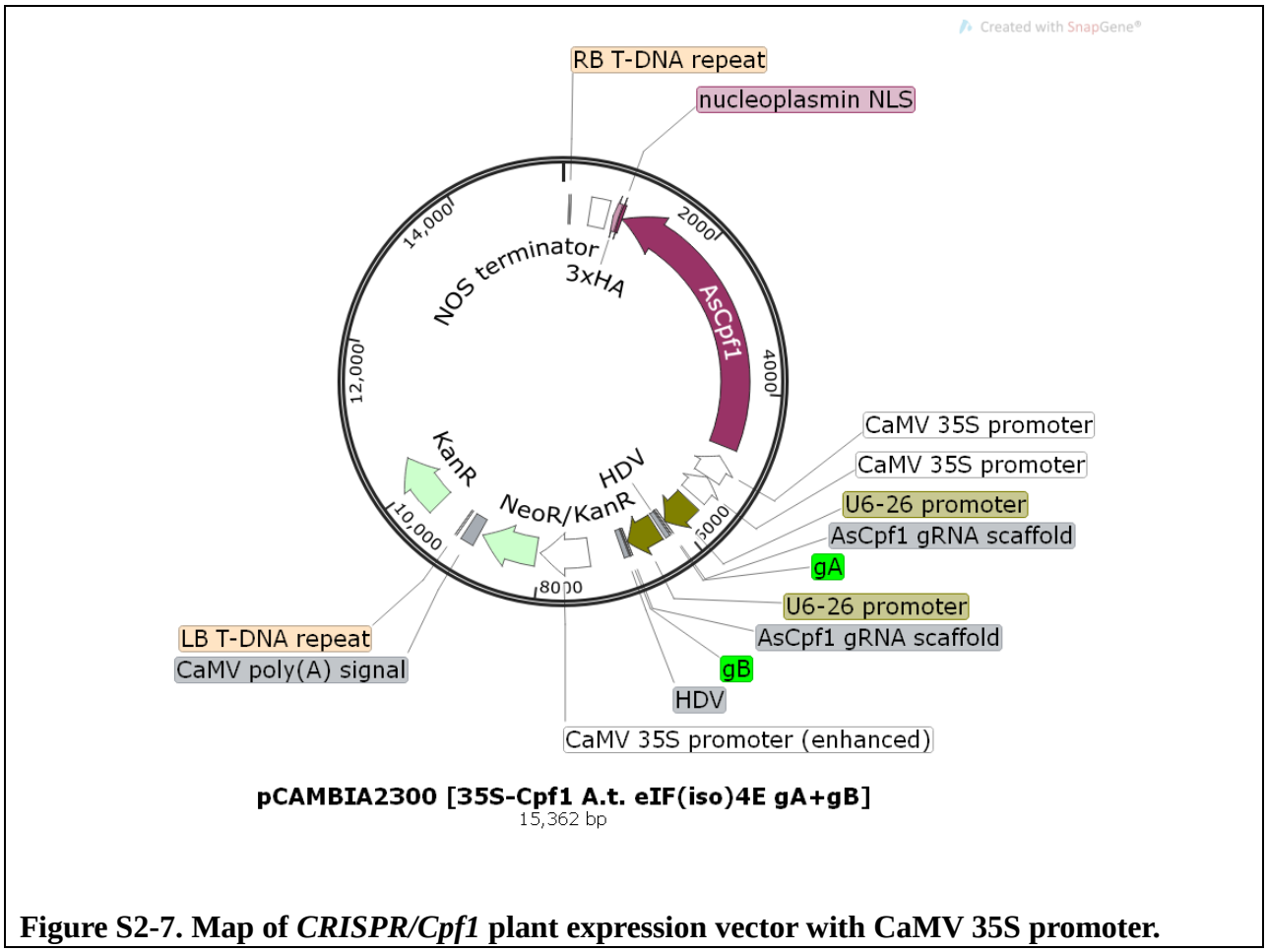

Figure S2-7. Map of *CRISPR/Cpf1* plant expression vector with CaMV 35S promoter.

Figure S2-8. Sequence of *CRISPR/Cpf1* plant expression T-DNA with CaMV 35S promoter.

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TAGAAAAGGAAGGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCC
CAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGA
TATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCTTCCTCTATATAAGGAAGTTCATTTCATTTGGA
GAGGACACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCGAGCTTTCGCAGATCTGTCGATCGACCATGGGGATTGA
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TACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGG
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TAATTCGGCGTTAATTCAGTACATTAAAAACGTCCGCAATGTGTTATTAAGTTGTCTAAGCGTCAATTTGTTTACACCACAATATA
TCCTGCCA

3. Simultaneous CRISPR/Cas9-mediated editing of cassava *eIF4E* isoforms *nCBP-1* and *nCBP-2* confers elevated resistance to cassava brown streak disease

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Background

Cassava brown streak disease (CBSD) is a threat to food and economic security for smallholder farmers in sub-Saharan Africa. First reported in the 1930s in lowland and coastal East Africa, CBSD has since spread west to higher altitudes in Uganda, Kenya, Tanzania, Burundi, and the Democratic Republic of Congo (Adams *et al.*, 2013; Alicai *et al.*, 2007; Bigirimana *et al.*, 2011; Mbanzibwa *et al.*, 2011; Mulimbi *et al.*, 2012). The CBSD vector is the whitefly *Bemisia tabaci*, which has a broad geographical distribution across sub-Saharan Africa (Legg *et al.*, 2014). CBSD symptoms include leaf chlorosis, brown streaks on stems, and necrosis of the storage roots. CBSD immunity, or complete non-infection of the cassava plant (*Manihot esculenta* Crantz), has not been observed within farmer cultivars (Kaweesi *et al.*, 2014). Infection can occur in resistant cultivars such as Kaleso and Namikonga, but multiplication, movement, and disease symptoms are limited (Kaweesi *et al.*, 2014). Tolerant cultivars Nachinyaya and Kiroba can be infected and support virus movement and replication, but with intermediate symptoms, while susceptible cassava cultivars 60444 and Albert support high levels of virus and develop severe CBSD symptoms (Hillocks *et al.*, 2001; Maruthi *et al.*, 2014; Masiga *et al.*, 2014; Ogwok *et al.*, 2015). Since symptoms may be subtle or develop only within the underground storage roots, CBSD may claim an entire crop without the farmer's knowledge until harvest (Legg *et al.*, 2015; Patil *et al.*, 2015). Necrotic lesions render the storage roots unfit for market and human consumption with losses of up to 70% root weight reported (Hillocks *et al.*, 2001). The International Institute of Tropical Agriculture (IITA) estimated that CBSD causes $175 million loss in East Africa each year (Michael, 2013).

The causative agents of CBSD, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), belong to the family *Potyviridae* (Genus: *Ipomovirus*) (Revers and García, 2015). These non-enveloped, flexuous, filamentous viruses contain a positive-sense, single-stranded RNA, with a 3'-poly(A) terminus (King *et al.*, 2012). CBSV recruits host cellular translation machinery to produce a polyprotein of 2902 amino acids that is proteolytically cleaved into 10 mature proteins (Mbanzibwa *et al.*, 2009). A viral genome-linked (VPg) protein is covalently linked to the 5' end of the viral genome and is required for infection by this pathogen (Robaglia and Caranta, 2006; Wang and Krishnaswamy, 2012).

We aimed to apply the CRISPR/Cas9 technology to knockout the VPg-associated cassava eIF4E isoform(s). This approach to engineering potyvirus resistance has been successfully demonstrated in *A. thaliana* and cucumber (Chandrasekaran *et al.*, 2016; Pyott *et al.*, 2016). Here, we show that targeted mutagenesis of specific cassava *eIF4E* isoforms *nCBP-1* and *nCBP-2* by the CRISPR/Cas9 system reduces levels of CBSD symptoms and CBSV accumulation in storage roots. Simultaneous disruption of both *nCBP* isoforms resulted in a larger decrease in disease symptoms than disruption of either isoform individually.

Results

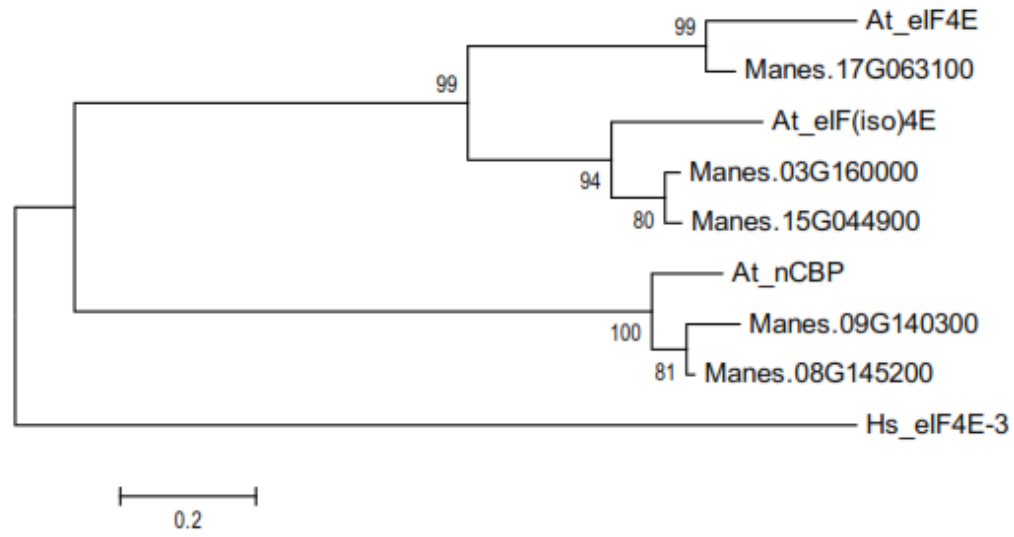
Identification and sequence comparison of eIF4E isoforms in cassava varieties

To identify the eIF4E family protein(s), a BLAST search of the AM560-2 cassava cultivar genome (assembly version 6.1) was done via Phytozome using *A. thaliana* eIF4E family proteins as the queries (Bredeson *et al.*, 2016; Goodstein *et al.*, 2012). Five cassava proteins were found that phylogenetically branched with the eIF4E, eIF(iso)4E, and nCBP sub-groups (Figure 3-1a). Two of the cassava eIF4E family proteins joined within the eIF(iso)4E sub-group, and another two joined within the nCBP sub-group. This is in agreement with findings by Shi *et al.* (2017). Percent identity analysis further supported this grouping as the eIF(iso)4E- and nCBP-similar proteins had high amino acid identity (Figure 3-1b). Based upon this phylogenetic analysis, one *eIF4E*, two *eIF(iso)4E*, and two *nCBP* genes cassava genes were re-named according to their sub-groups, as described in Figure 3-1c.

nCBP-1 and nCBP-2 isoforms interact with CBSV and UCBSV VPg in yeast

To identify the interaction partner(s) for the CBSV and UCBSV VPgs, a yeast two-hybrid system was used to assess the VPg-eIF4E isoform interactions. The VPg proteins from CBSV Naliendele isolate TZ:Nal3-1:07 (CBSV-Nal) and UCBSV isolate UG:T04-42:04 (UCBSV-T04) were fused to the B42 activation domain and transformed into yeast strain EGY48. All five cap-binding proteins were fused to the LexA DNA-binding domain and transformed into VPg yeast lines. Likewise fused, TuMV VPg and *A. thaliana* eIF(iso)4E were transformed into yeast as a positive control, and empty vectors were transformed as negative controls. Five colonies from each transformation were plated on selective media supplemented with X-gal. In this assay, a blue color is indicative of protein-protein interaction dependent activity of the β-galactosidase reporter. Based upon high amino acid sequence identity within eIF4E-family subgroups, we hypothesized both members of a sub-group would interact with CBSV and UCBSV VPgs. Both nCBP-1 and nCBP-2 showed strong interactions with the VPgs, visually comparable to the positive control (Figure 3-2). *nCBP-1* and *nCBP-2* were selected for CRISPR/Cas9-mediated editing to abolish the critical VPg-eIF4E family protein interaction.

a.



b.

	Hs_eIF4E-3	At_nCBP	Manes.08G145200	Manes.09G140300	At_eIF4E	Manes.17G063100	At_eIF(iso)4E	Manes.03G160000	Manes.15G044900
Hs_eIF4E-3	100.00								
At_nCBP	30.46	100.00							
Manes.08G145200	30.29	77.38	100.00						
Manes.09G140300	30.86	74.09	88.34	100.00					
At_eIF4E	29.48	29.49	30.80	28.77	100.00				
Manes.17G063100	31.21	28.37	29.73	28.11	68.24	100.00			
At_eIF(iso)4E	29.17	31.55	31.05	29.95	47.67	50.26	100.00		
Manes.03G160000	33.53	30.89	30.93	30.89	50.25	53.33	69.43	100.00	
Manes.15G044900	33.53	31.41	30.41	30.89	49.75	55.38	69.43	92.50	100.00

c.

Locus Name	Description
Manes.17G063100	eIF4E
Manes.03G160000	eIF(iso)4E-1
Manes.15G044900	eIF(iso)4E-2
Manes.09G140300	nCBP-1
Manes.08G145200	nCBP-2

Figure 3-1. Identification of cassava eIF4E family homologs. (a) Phylogenetic relationships of cassava eIF4E family with *A. thaliana*, At, eIF4E family inferred by the Maximum Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Tree rooted to *Homo sapiens*, Hs, eIF4E-3. (b) Percent identity matrix of cassava eIF4E family with At eIF4E family using amino acid sequences. (c) Descriptions of cassava eIF4E family based upon phylogenetic relationships and percent identity matrix.

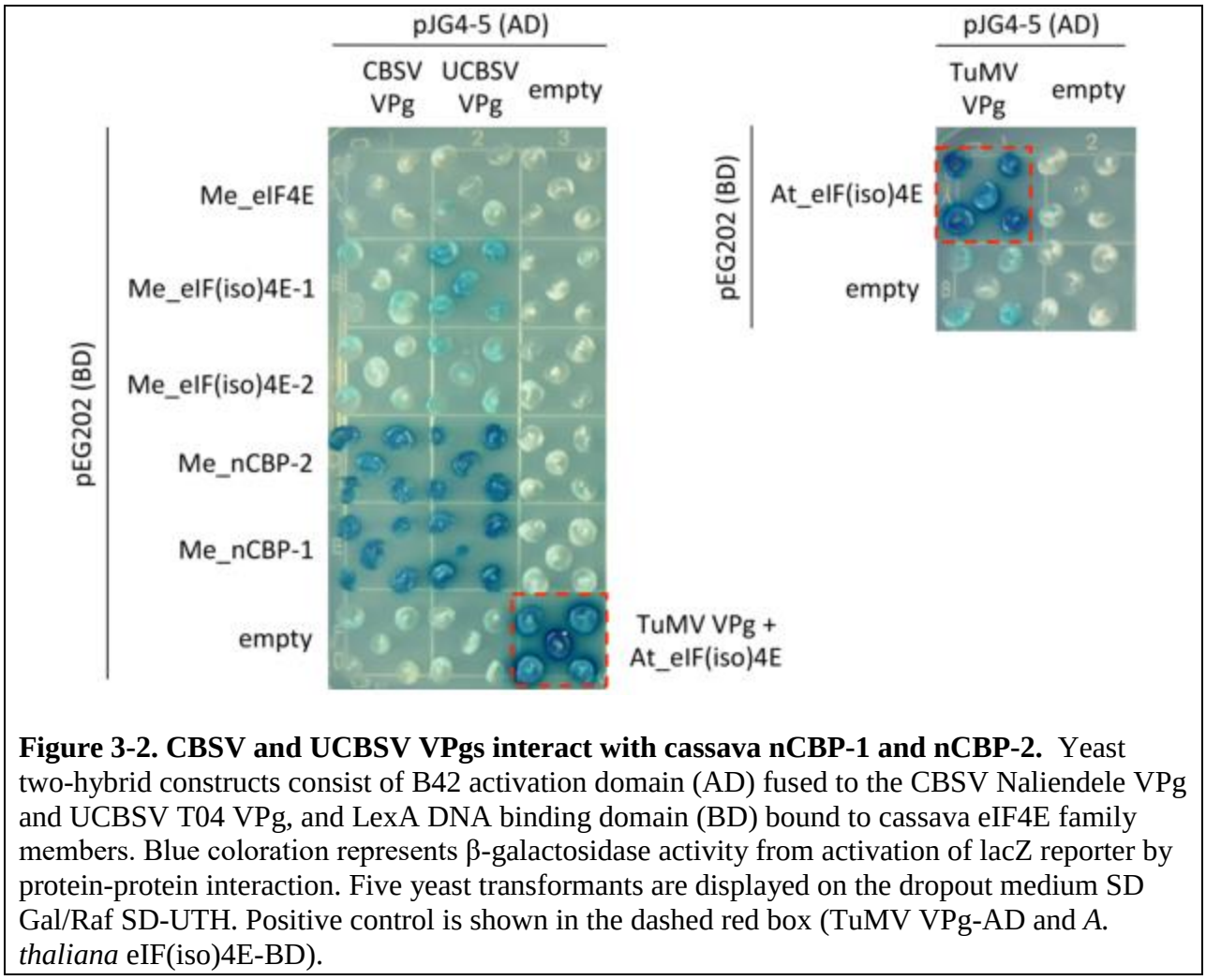


Figure 3-2. CBSV and UCBSV VPgs interact with cassava nCBP-1 and nCBP-2. Yeast two-hybrid constructs consist of B42 activation domain (AD) fused to the CBSV Naliendele VPg and UCBSV T04 VPg, and LexA DNA binding domain (BD) bound to cassava eIF4E family members. Blue coloration represents β-galactosidase activity from activation of lacZ reporter by protein-protein interaction. Five yeast transformants are displayed on the dropout medium SD Gal/Raf SD-UTH. Positive control is shown in the dashed red box (TuMV VPg-AD and *A. thaliana* eIF(iso)4E-BD).

Site-specific mutation of *eIF4E* isoforms by transgenic expression of sgRNA-guided Cas9

CRISPR/Cas9 was employed to generate mutant alleles of cassava *eIF4E* isoforms. Seven constructs were assembled to target various sites in *nCBP-1*, *nCBP-2*, and *eIF4E* (Table 3-1). *Agrobacterium* carrying these constructs were then used to transform friable embryogenic calli (FEC) derived from cassava cultivar 60444 (Figure 3-3). Transgenic T0 plants were selected in tissue culture using the *npt*II selectable marker in order to recover plants in which the CRISPR/Cas9 reagents had been integrated into the plant genome. Multiple independent T0 transgenic plant lines were recovered for each construct (Table S3-1). Sites in each *nCBP* gene were targeted to disrupt restriction enzyme recognition sequences (Figure S3-2). Restriction digestion done on a PCR product from T0 plants using restriction enzyme *Sml*I indicated successful mutagenesis of both *nCBP* genes (Figure S3-2). Cassava is diploid, carrying two copies of each *nCBP* gene. Absence of the wild-type digested product indicates that both alleles were successfully mutagenized.

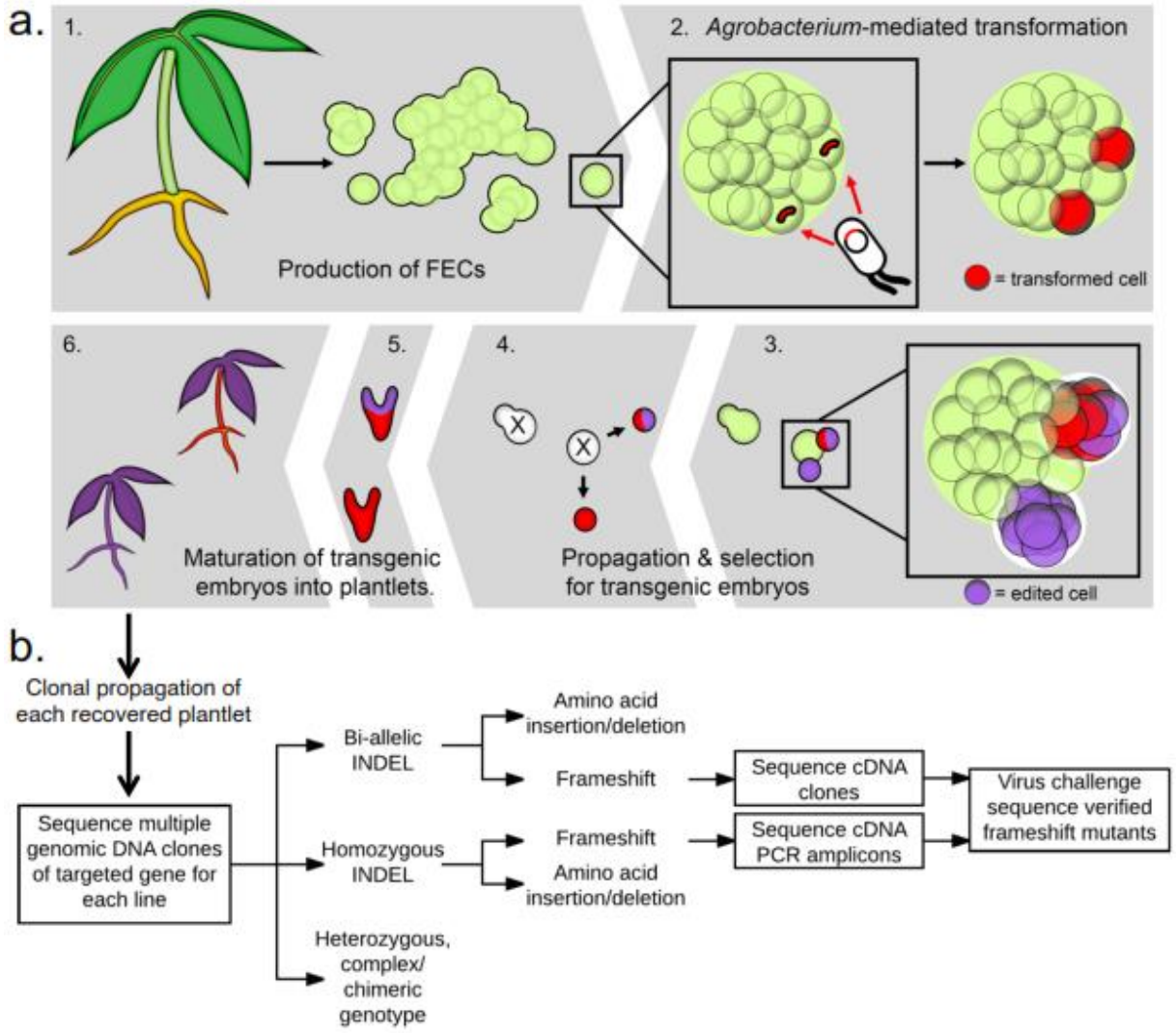


Figure 3-3. Method for generating CRISPR/Cas9 mediated gene edited cassava plants. (a) Transgenic cassava are produced via *Agrobacterium*-mediated transformation of friable embryogenic callus (FECs). 1) FECs are induced from somatic tissues by placing the latter on growth media supplemented with picloram. FECs are comprised of aggregated spheroid embryogenic units. Individual units (boxed in panel 1 and enlarged in panel 2) range from a few cells to approximately 1 mm in diameter. 2) FECs are transformed with CRISPR/Cas9 constructs through co-culture with *Agrobacterium tumefaciens.* Red semi-circles denote T-DNA fragments and red spheres denote transformed cells. 3) Cells on the surface of embryogenic units, transformed or untransformed, divide to produce new embryogenic units. CRISPR/Cas9 editing can occur prior to or after division. Edited cells are colored purple. 4) Antibiotic selection kills untransformed daughter embryoids. Dead cells marked with "X". Transformed embryogenic units are spread over selective media and form colonies. One mature embryo per colony is recovered (5), and develops into a plantlet (6). Each regenerated plant is clonally propagated and referred to as a mutant line. (b) Workflow for mutant genotype characterization and line selection.

The range of mutations generated in each transgenic plant was analyzed by subcloning and sequence analysis, revealing an array of homozygous, bi-allelic, heterozygous, complex, and wild-type genotypes (Table S3-1). Bi-allelic mutations contained different mutations on the two alleles. Heterozygous plants carried one mutagenized allele and one wild-type allele. Plants were considered complex if they carried more than two sequence patterns, strongly suggesting chimerism (Odipio *et al.,* 2017; Zhang *et al.,* 2014). The genotypes of edited plants had Cas9-induced INDELs ranging from insertions of 1 to 16 bp and deletions as large as 127 bp (Table S4-1). Review of all genotyped plants revealed that 13/55 (24%) carried homozygous mutations, 31/55 (56%) carried bi-allelic mutations, 1/55 (2%) were heterozygous, 5/55 (10%) were complex, and another 5/55 (10%) were wild-type genotypes (Table 3-1). In total, 80% of plants contained either homozygous or bi-allelic mutations, and CRISPR/Cas9 activity was observed in 91% of the plants studied.

Sequence analysis of INDEL-induced frameshifts in *nCBP*s identifies unpredicted *ncbp-1* splice variants

Given the yeast two-hybrid interaction between the nCBPs and viral VPg proteins, we chose to test the effects of mutations in *nCBP-1* and *nCBP-2* individually, as well as both *nCBP*s in tandem, in CBSV and UCBSV disease trials in a greenhouse. Lines with homozygous mutations in exon 1 were prioritized (Figure 3-3). The mutant lines chosen for these trials, *ncbp-1*, *ncbp-2*, *ncbp-1/2* #2, and *ncbp-1/2* #8, each had an INDEL at the 3' end of the first exon of each targeted gene (Figure 3-4). The INDELs either directly resulted in a frameshift or disrupted the exon-intron junction so that an out of frame splice variant was predicted to be produced (Figure S3-3). To further characterize these mutations, cDNA clone sequencing (clone-seq) was done (Figures 3-3, 3-5). The homozygous *ncbp-1* allele from *ncbp-1/2* #2 was also analyzed for comparison. Of nine *ncbp-1* clones from *ncbp-1/2* #2, eight displayed the wild-type splicing pattern (referred to as type 1) with the A-insertion predicted from genomic DNA sequence results. This generates a frameshift. One splice variant (referred to as type 2) was also observed (Figure 3-5). This alternative splice form results in an insertion of 35 nucleotides but does not shift the reading frame. Thus, this splice variant encodes a full protein with a 12 amino acid internal insertion. This splicing pattern was not observed in any wild-type *nCBP-1* clones, however, may occur at low frequency.

Clone-seq analysis of seven *ncbp-1* clones from mutant line *ncbp-1/2* #8 cDNA similarly found predicted INDELs. Two clones displayed the wild-type (type 1) splicing pattern and the predicted A-deletion that alters the reading frame. Four clones showed a sequence pattern that suggests a third splicing variant (type 3) at an upstream alternative splice site (Figure 3-5) (Reddy *et al.,* 2007). Both observed cDNA sequence patterns are frameshifted.

Construct	Gene Target	Total # of lines	Homozygous	Bi-allelic	Heterozygous	Chimeric	WT
BS01	*nCBP-1*	6	2	1	0	2	1
BS02	*nCBP-2*	6	1	3	0	2	0
BS03	*nCBP-1*	10	4	5	0	0	1
BS04	*nCBP-2*	15	2	10	1	1	1
BS05	*nCBP-1/2*	8	1	6	0	0	1
BS06	*eIF4E*	7	3	4	0	0	0
BS07	*eIF4E*	3	0	2	0	0	1
	Total	*55*	*13*	*31*	*1*	*5*	*5*
	Percent	*100%*	*24%*	*56%*	*2%*	*9%*	*9%*
	Combined Percentages		*91%*				
			80%				

Table 3-1. Genotype counts of transgenic T_0 cassava lines.

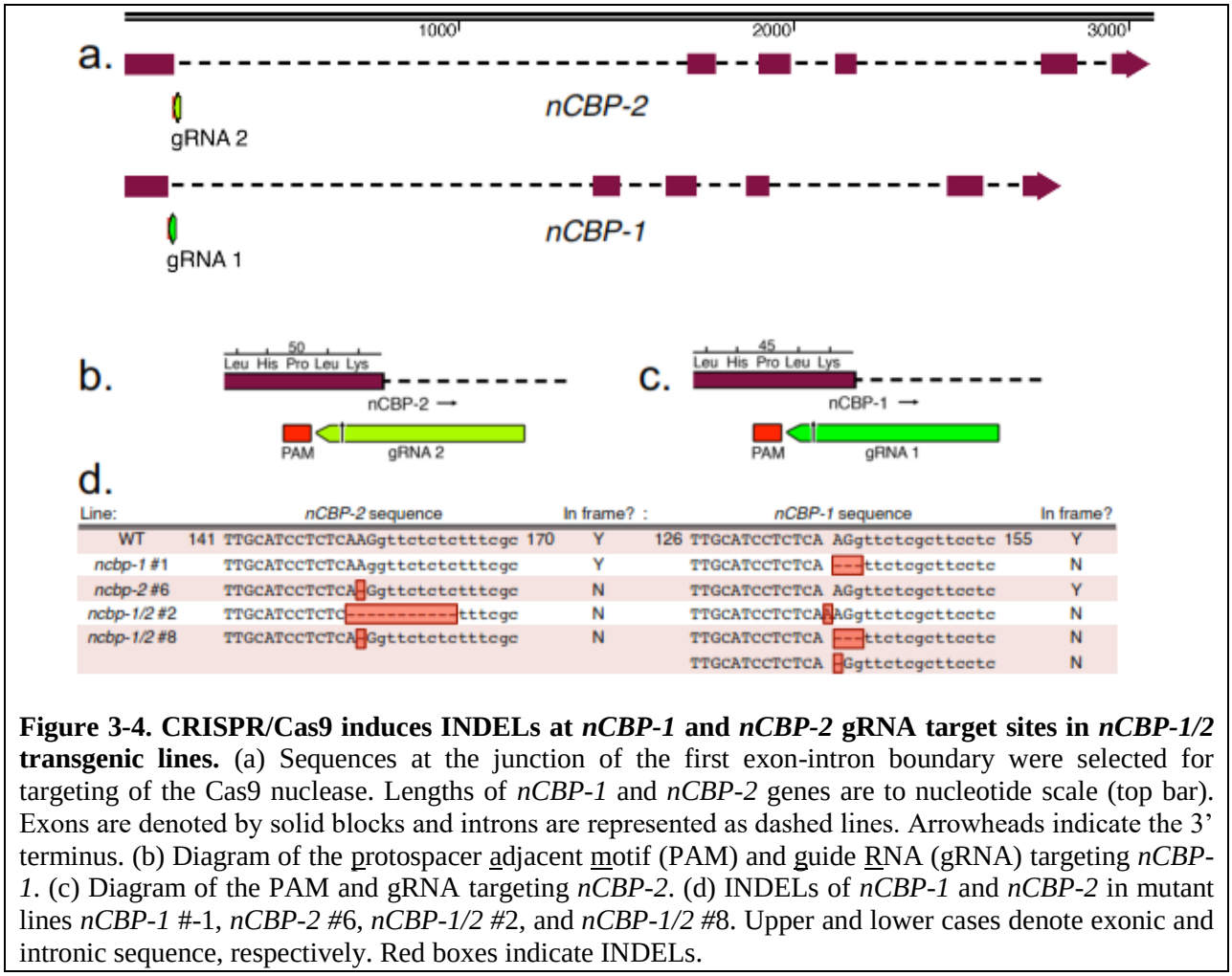


Figure 3-4. CRISPR/Cas9 induces INDELs at *nCBP-1* and *nCBP-2* gRNA target sites in *nCBP-1/2* transgenic lines. (a) Sequences at the junction of the first exon-intron boundary were selected for targeting of the Cas9 nuclease. Lengths of *nCBP-1* and *nCBP-2* genes are to nucleotide scale (top bar). Exons are denoted by solid blocks and introns are represented as dashed lines. Arrowheads indicate the 3' terminus. (b) Diagram of the protospacer adjacent motif (PAM) and guide RNA (gRNA) targeting *nCBP-1*. (c) Diagram of the PAM and gRNA targeting *nCBP-2*. (d) INDELs of *nCBP-1* and *nCBP-2* in mutant lines *nCBP-1* #-1, *nCBP-2* #6, *nCBP-1/2* #2, and *nCBP-1/2* #8. Upper and lower cases denote exonic and intronic sequence, respectively. Red boxes indicate INDELs.

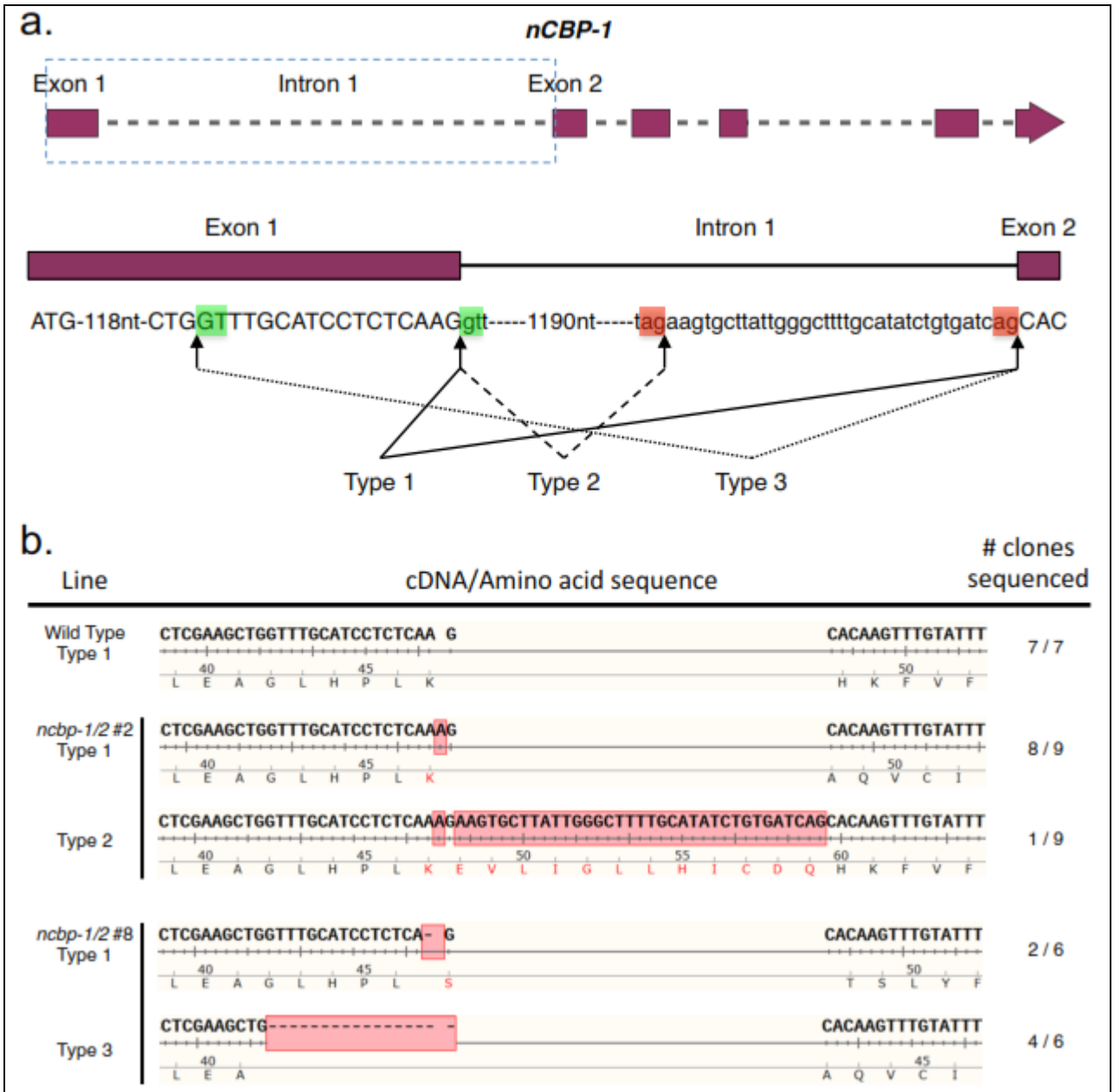


Figure 3-5. Alternative splicing of *nCBP-1* generates variants that maintain the downstream reading frame.(a) Schematic of canonical and alternative nCBP-1 splice sites. Boxed region of the nCBP-1 gene model is enlarged below. Exon and intron sequences are given in capital and small letters, respectively. Green and red boxes highlight splice motifs at the 5' and 3' end of introns, respectively. Type 1 splicing produces the predicted wild type nCBP-1 cDNA sequence. Type 2 and 3 splicing are observed in ncbp-1/2 lines #2 & #8, respectively. (b) cDNA sequences detected in clone-seq experiments. Red boxes denote INDELs resulting from both CRISPR/Cas9-mediated edits and alternative splicing. In ncbp-1/2 #2, type 2 splicing results in retention of 3' sequence from intron 1 of one ncbp-1 allele (1 of 9 clones sequenced). In ncbp-1/2 #8, an INDEL disrupting the canonical splice motif between exon 1 and intron 1 of ncbp-1 results in a type 3 splice variant (4 of 6 clones sequenced).

ncbp-1/ncbp-2 double mutants exhibited reduced UCBSV symptom incidence and slowed CBSV symptom onset

CBSV has been described as being more virulent than UCBSV (Kaweesi *et al.*, 2014; Mohammed *et al.*, 2012; Ogwok *et al.*, 2015). Challenge with a stronger pathogen may mask subtle phenotypes that could be presented during challenge with the weaker pathogen. As such, three disease trials for each virus species were carried out. The *ncbp-1, ncbp-2, ncbp-1/2 #2, ncbp-1/2* #8, and wild-type 60444 plants were chip-bud graft inoculated with either CBSV-Nal or UCBSV-T04 (Wagaba et. al, 2013). Aerial disease incidence was scored every week for 12 to 14 weeks after grafting. This analysis describes the percentage of plants showing any level of foliar or stem symptoms at each time point. At least five replicate plant clones were included for each genotype ($n \geq 5$). Inoculation with UCBSV-T04 did not produce stem symptoms. Consequently, only foliar disease incidence was recorded for those trials. Fluctuations in the percentage of plants that exhibited symptoms at each time point (% incidence) results from the shedding of symptomatic leaves throughout the experiment. Disease incidence for each given genotype varied across experimental replicates, possibly due to variance in viral load of the chip-bud donor or a change in environmental conditions affecting disease pressure. However, a consistent relationship between genotypes was observed. Across all three experimental replicates, *ncbp-1/ncbp-2* double mutants exhibited reduced symptom incidence relative to wild-type plants and *ncbp-1*. The *ncbp-2* phenotype was intermediate between the double mutant and wild-type incidence rates (Figures 3-6b, S3-4). Aerial UCBSV virus titer was measured for one experiment, but proved to be highly variable across biological replicates (Figure S3-9).

In challenges with CBSV-Nal, wild-type plants produced strong foliar and stem symptoms in contrast to the UCBSV trials. Across all three experimental replicates, *ncbp-1/ncbp-2* double mutants exhibited delayed symptom development relative to wild type and *ncbp-1* (Figures 3-6a, S3-4). In two experiments the double mutant lines reached 100% incidence at a markedly reduced rate relative to wild type and *ncbp-1*; in the remaining experiment, the same lines never rose above 43% incidence. *ncbp-2* exhibited symptom incidence development similar to wild type and *ncbp-1* in two experiments and displayed an intermediate phenotype in the third experiment (Figure6b, S4a, S4b).

ncbp-1/ncbp-2 lines exhibit reduced aerial symptom severity after challenge with CBSV

For the described CBSV challenges, combined leaf-stem scores were also used to track aggregate aerial CBSD severity for each genotype over time (Table 3-2, Figures 3-6c, S3-5). Wild-type, *ncbp-1*, and *ncbp-2* plants displayed similar levels of disease, although in one of three experiments *ncbp-2* developed statistically significantly less severe symptoms than wild type or *ncbp-1* (Figure S3-5a). This experiment was the same one in which *ncbp-2* symptom incidence was intermediate between wild type/*ncbp-1* and *ncbp* double mutant levels (Figure S3-4c). The *ncbp-1*/*ncbp-2* double mutants had greatly reduced CBSD severity in all three trials. Area under the disease progression curve (AUDPC) analysis revealed the reduced aerial symptom severity in *ncbp-1*/*ncbp-2* double mutants to be statistically significant in all three experimental replicates (Figures 3-6d, S3-5). While *ncbp-1*/*ncbp-2* stem symptom severity was reduced in all three experiments, average leaf symptom severity tracked closely with wild type in one experiment (Figures 3-7, S3-6, S3-7). Despite this, it is clear that mutating both *nCBP* isoforms had an effect on CBSD disease development.

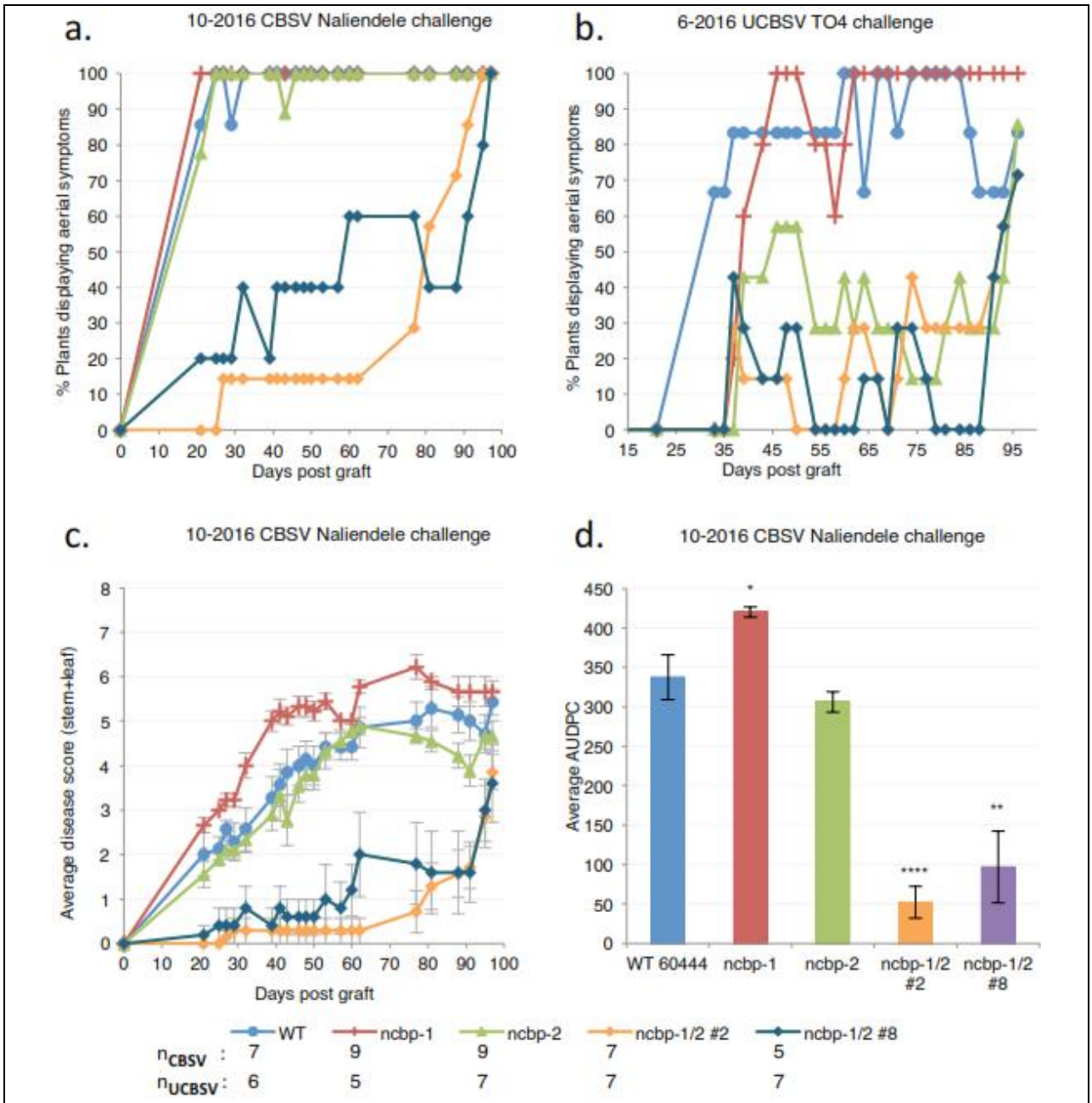


Figure 3-6. *ncbp-1/ncbp-2* double mutants exhibit delayed CBSV symptom onset and reduced symptom severity. (a), (b) Aerial symptom incidence reported as percent of wild type, *ncbp-1*, *ncbp-2*, or *ncbp-1/ncbp-2* plants (n≥5) bud-graft inoculated with either CBSV Naliendele or UCBSV T04 isolates, respectively. *ncbp-1/ncbp-2* double mutant lines #2 and #8 are the product of independent transgenic events. (c) Disease progression curves for previously described CBSV inoculated plants. Leaf and stem symptoms were each scored on a 0-4 scale and summed to obtain an aggregate aerial score. (d) Average area under the disease progression curve (AUDPC) derived from data plotted in (c). Error bars in (c) and (d) indicate standard error of the mean. Statistical differences were detected by Welch's t-test, α=0.05, *≤0.05, **≤0.01, ****≤0.0001.

Leaf symptoms	Score	Shoot symptoms
Asymptomatic	**0**	Asymptomatic
Specks of chlorosis localized to a small section of leaf	**1**	Punctate brown streaks localized to small length of stem
Widespread chlorosis throughout leaves	**2**	Spreading brown streaks along less than 10% of the stem
Widespread chlorosis accompanied by slight die-back of terminal branches	**3**	Brown streaking along 10-60% of stem
Widespread chlorosis and plant die-back	**4**	Continuous brown streaking along the entire stem length

Table 3-2. Aerial symptom scoring scale.

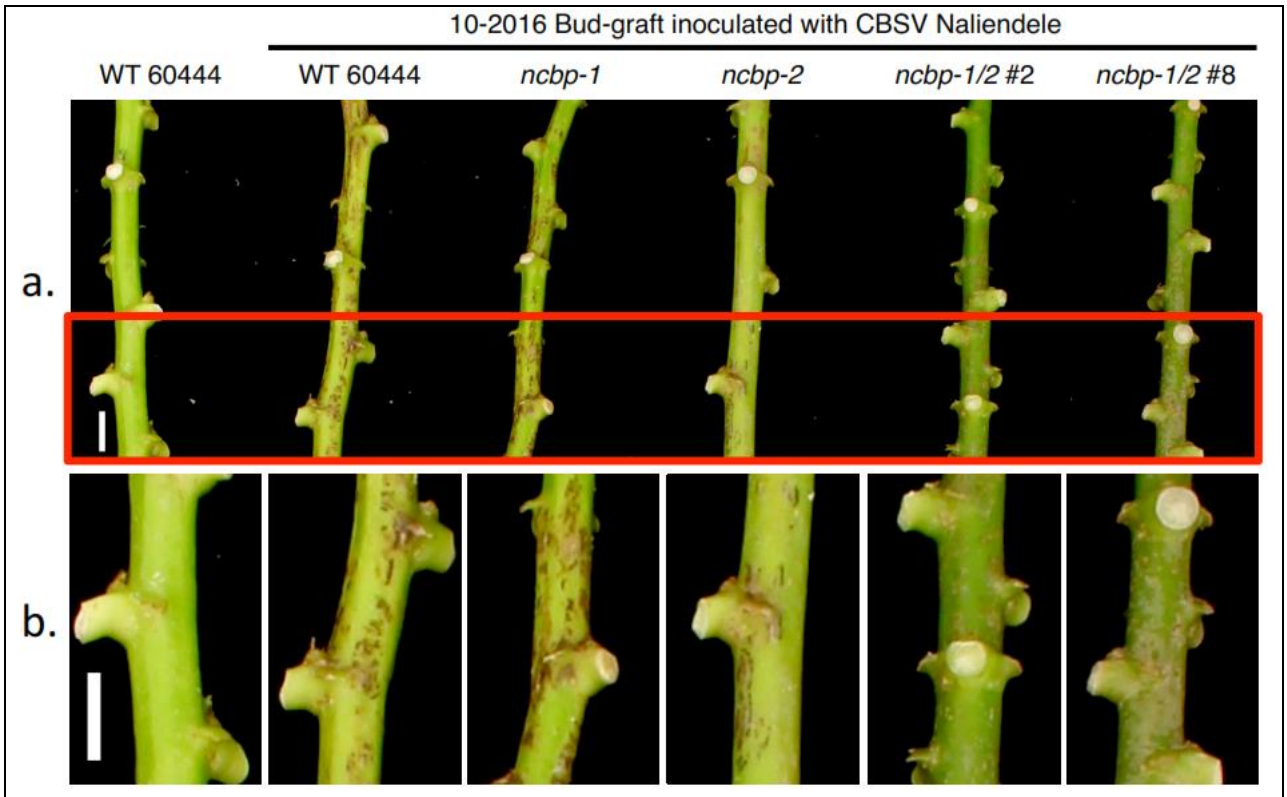


Figure 3-7. CBSD stem symptom attenuation on *ncbp-1/ncbp-2* double mutants. (a) Representative wild type, *ncbp-1*, *ncbp-2*, or *ncbp-1/ncbp-2* stems displaying varying degrees of brown streak symptoms 14 weeks post graft inoculation with CBSV Naliendele. *ncbp-1/ncbp-2* double mutants present reduced brown streaking and associated dark pigmentation along the length their stems. Portions of stems boxed in red are enlarged in (b). Imaged portions of stems are all approximately the same distance above the graft site.

ncbp-1/*ncbp-2* double mutant storage roots are less symptomatic and accumulate less virus

At 12 to 14 weeks after graft inoculation, storage roots were excavated and assessed for root necrosis. Only inoculation with CBSV-Nal produced storage root symptoms. Each storage root of a plant was divided into five sections and each section scored on a 1-5 scale for CBSD symptom severity (Figure 3-8a). Average symptom scores for each genotype were compared. *ncbp-2* and *ncbp-1*/*ncbp-2* mutant lines all exhibited significantly reduced symptom scores relative to wild type and *ncbp-1* (Figure 3-8b). Reverse transcription-quantitative polymerase chain reaction (qPCR) was used to measure CBSV-Nal RNA levels in *ncbp-1*/*ncbp-2* double mutants. Viral RNA levels in *ncbp-1*/*ncbp-2* roots were reduced 43-45% compared to wild-type roots (Figure 3-8c).

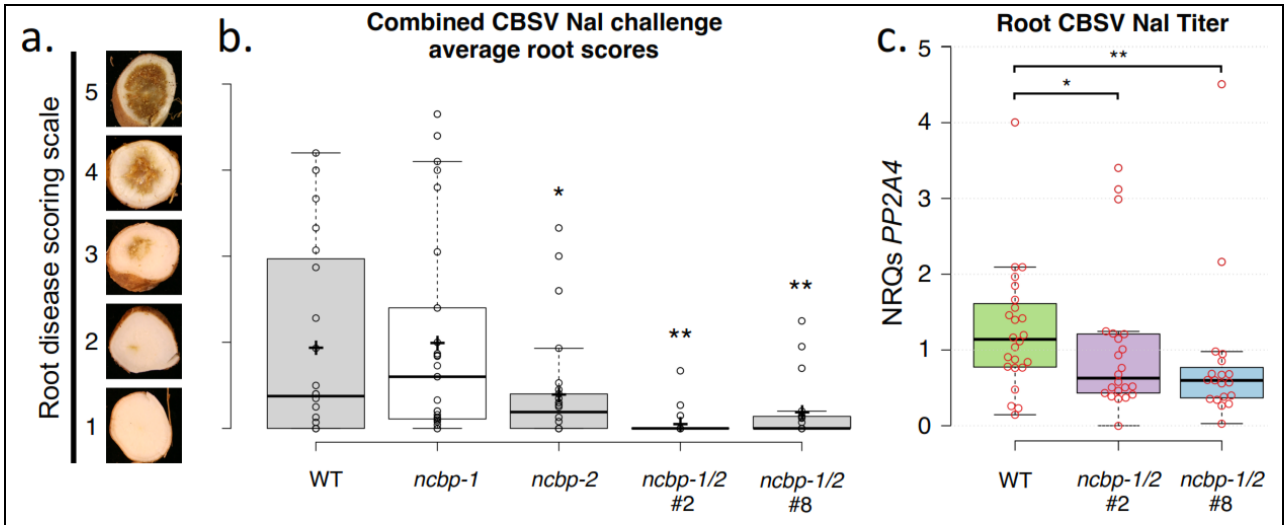


Figure 3-8. *ncbp-1/ncbp-2* double mutant storage roots are less symptomatic and accumulate less virus. (a) Storage root sections were assessed on a 1-5 scale, where 1 corresponds with no symptoms and 5 with extensive necrosis present throughout the diameter of root. (b) *ncbp-1/2* storage roots are significantly less symptomatic than wild type at 12 to 14 weeks post bud-graft inoculation with CBSV Naliendele isolate. Points represent average scores of all storage root sections from a single plant. Data from three experimental replicates were pooled. Whiskers span the interquartile range, solid bars indicate the median of scores, + indicates the mean of scores. Statistical significance was detected by Welch's t-test, n≥19, α=0.05. (c) Quantitative real time PCR analysis reveals that *ncbp-1/2* storage roots accumulate less virus than wild type. CBSV *HAM1-LIKE* was normalized to *PP2A4*. Data from three experimental replicates were pooled. Significant differences were detected with a Mann-Whitney U-test, n≥19, α=0.05, *≤0.05, **≤0.01, ****≤0.0001.

Discussion

The CRISPR/Cas9 system has emerged as a powerful tool for plant genome editing and rapid crop improvement. In the context of disease resistance in crop species, this system has been employed to target *mildew-resistance locus O* (*MLO*) in wheat, and generate broad potyvirus resistance in cucumber by disrupting function of the e*IF4E* gene (Chandrasekaran *et al.*, 2016; Wang *et al.*, 2014). In the present study, we targeted the *nCBP*s to assess their putative function as CBSD susceptibility factors in cassava.

Previous studies have shown that host eIF4E and viral VPg interaction is necessary for potyviral infection (Ashby *et al.*, 2011; Charron *et al.*, 2008; Kang *et al.*, 2005; Leonard *et al.*, 2000; Yeam *et al.*, 2007). We identified five eIF4E family members in cassava, corroborating a recent analysis by Shi *et al.* (2017). Cassava is thought to be an ancestral allopolyploid, likely yielding the two *eIF(iso)4E* and *nCBP* genes (Fregene *et al.*, 1997). The presence of multiple eIF4E isoforms may indicate sub-functionalization and specialization in translational control of differently methylated mRNA cap structures, or confer some functional redundancy that eases constraints on eIF4E evolution for potyvirus resistance (Carberry *et al.*, 1991; Charron *et al.*, 2008; Moury *et al.*, 2014). Attempts to identify markers associated with CBSD resistance indicate that multiple loci are involved, and transcriptional analyses suggest the contribution of hormone signaling pathways (Maruthi *et al.*, 2014; Masumba *et al.*, 2017). Examination of CBSD-resistant, -tolerant, and -susceptible cultivars by Shi and colleagues also found that these categories are not associated with *eIF4E* family single nucleotide polymorphisms (Shi *et al.*, 2017). As such, a biochemical study of the VPg and eIF4E family interaction was essential to identify a potential susceptibility gene(s).

Yeast two-hybrid analysis showed strong interactions between the nCBPs and the CBSV-Naliendele and UCBSV-T04 VPg proteins, to levels visually equivalent to the positive control TuMV VPg-*A. thaliana* eIF(iso)4E interaction (Fig. 2). First identified in *A. thaliana*, nCBP has a distinct amino acid sequence and exhibits methylated-cap-binding property (Kropiwnicka *et al.*, 2015; Ruud *et al.*, 1998). To date, there is no precedent for recruitment of nCBPs by VPg proteins belonging to the family *Potyviridae*. However, this isoform has been identified as a novel recessive resistance gene toward viruses in the *Alphaflexiviridae* and *Betaflexiviridae* families (Keima *et al.*, 2017). In the case of potexvirus *Plantago asiatica mosaic virus* (PlAMV), nCBP loss in *A. thaliana* impaired viral cell-to-cell movement by inhibiting accumulation of viral movement proteins from a subgenomic RNA. It is unclear if *A. thaliana* nCBP is either required for subgenomic RNA stability or translation of PlAMV movement proteins. In contrast, there is evidence that many members of *Potyviridae* produce the potyvirus P3N-PIPO movement protein through RNA polymerase slippage (Hagiwara-Komoda *et al.*, 2016; Olspert *et al.*, 2015; Rodamilans *et al.*, 2015). As such, while nCBP may similarly play a critical role in the accumulation of the CBSV movement protein, the underlying mechanism is likely to be different from those used during *Alpha-* and *Betaflexavirdae* infection. It has also been found that distantly related potyviruses that infect a common host may utilize different eIF4E isoforms for movement (Contreras-Paredes *et al.*, 2013; Eskelin *et al.*, 2011; Gao *et al.*, 2004; Miras *et al.*, 2017). Furthermore, evidence suggests that some potyviruses may utilize one specific isoform for translation and another distinct isoform for movement (Contreras-Paredes *et al.*, 2013; Gao *et al.*, 2004). This complexity makes it difficult to predict what roles cassava nCBPs may have in

the CBSV life cycle. Further study is required to characterize the role of nCBP in translation, genome stability, and viral movement processes.

Five CRISPR/Cas9 expression constructs were designed and transgenically integrated into the cassava genome to target the *nCBP* genes individually and simultaneously. In transgenic plant lines, mutations were detected by restriction enzyme site loss analysis and Sanger sequencing. We observed homozygous, bi-allelic, heterozygous, complex, and wild-type genotypes. Homozygous mutations may have been generated by identical NHEJ repair, or homologous recombination-based repair from the opposite allele. Considering the low incidence of the latter in plants, identical NHEJ repair may be more likely (Peng *et al.*, 2016). While transgenic plants derived from FECs are thought to be of single cell origin (Fig. 3a), reducing the likelihood of transgenic chimeras (Schreuder *et al.*, 2001; Taylor *et al.*, 1996), Odipo et al. (2017) have reported the production of chimeric plants via CRISPR/Cas9-mediated gene editing of phytoene desaturase in cassava. In depth analyses of lines with complex genotypes were not pursued, but they are likely chimeras resulting from Cas9/sgRNA activity being delayed until after embryogenic units began to replicate (Odipio *et al.*, 2017; Zhang *et al.*, 2014). Integrating CRISPR/Cas9 constructs into the cassava genome proved to be efficient for achieving gene editing as 91% of the transformed plant lines carried INDELs at the target sites, and desired homozygous and bi-allelic mutations were observed in 80% of plant lines. These frequencies compare favorably to previous CRISPR/Cas9-mediated mutagenesis studies in cassava, rice and tomato (Ma *et al.*, 2015; Odipio *et al.*, 2017; Pan *et al.*, 2016). Homozygous and bi-allelic genotype frequencies in rice and tomato were approximately 80% and 19%, respectively.

CBSD inoculation experiments were limited to plant lines carrying homozygous and bi-allelic mutations that resulted in a frameshift or disrupted the exon-intron junction, thus resulting in the production of frameshifted splice variants. Single-*nCBP* and double-*nCBP* mutant lines were challenged with isolates CBSV-Naliendele and UCBSV-T04 (Beyene *et al.*, 2017; Wagaba *et al.*, 2013). Levels of resistance to CBSD were strongly correlated with disrupting function of both *nCBP* genes. Over the course of 12 to 14 weeks, double-*nCBP* mutant lines exhibited delayed CBSD aerial (combined leaf and stem) symptom onset and reduced severity. Full resistance to CBSD was not achieved as some brown streaking of the stem occurred in double mutants, and leaf symptom severity tracked closely with wild type in our last experiment. Furthermore, toward the end of each challenge, aerial symptom incidence in the double-*nCBP* mutants approached wild-type levels (Fig. 6, Fig. S4). Single-*nCBP* mutants were generally not significantly different from the susceptible wild-type plants in response to CBSV-Nal challenges, but symptom incidence for *ncbp-2* fluctuated between wild-type and double mutant levels across UCBSV-T04 challenges. These results could be due to UCBSV being less virulent than CBSV (Kaweesi *et al.*, 2014; Mohammed *et al.*, 2012; Ogwok *et al.*, 2015). nCBP-2 may be more important for viral accumulation, and it could be that the mutant phenotype is masked by challenge with a more virulent pathogen or conditions conducive to high disease pressure. The latter may also influence inoculum concentrations in donor plants and result in the observed experiment-to-experiment variation in disease severity (Fig. S4, S5, S6, S7). This is consistent with observations that increases in *ncbp-2* symptom incidence to wild-type levels occurred when symptom incidence in *ncbp-1/ncbp-2* plants was elevated relative to other experiments. Variation in disease pressure may also explain the inconsistent leaf phenotype in the double-*nCBP* mutant plants. The mechanisms underlying CBSD leaf and stem symptom development are unknown

and it is possible that symptoms in different tissue types can be unequally influenced by varying levels of disease pressure.

At our challenge endpoints, symptom development and virus accumulation in the agronomically important tuberous roots were analyzed. Consistent with observations of aerial tissues, symptom severity in the roots was significantly lower in the double-*nCBP* mutants than in wild-type plants. CBSV titers in roots were significantly reduced in the double-*nCBP* mutants. Interestingly, the mutagenesis of *nCBP-2* resulted in reduced symptom severity as compared to wild-type plants and *ncbp-1* mutant lines. This result may be explained by the 10 fold higher expression of *nCBP-2* in the roots, but also highlights the possibility that nCBP-1 and nCBP-2 are not fully redundant (Fig. S8) (Wilson *et al.*, 2017). Assuming that the effects of *nCBP* mutations are due to the disruption of VPg-nCBP interactions, it is also possible that CBSV VPg may have a higher dependence for nCBP-2 than nCBP-1. Forcing CBSV to utilize less abundant isoforms, or those with suboptimal binding affinities, could attenuate CBSD progression. Additional transcriptional and biochemical studies will be needed to investigate these hypotheses.

Several explanations may account for the incomplete CBSD resistance of double-*nCBP* mutant cassava plants. First, unpredicted splice variants may have coded for proteins that were biologically functional for viral infection, at least in part (Fig. 5). The activation of normally silent, cryptic, splice sites is consistent with the intron definition of splicing (Lal *et al.*, 1999). Under this model, disruption of the wild-type splice site motifs, typically dinucleotides GU and AG at the 5' and 3' termini of introns, respectively, can activate cryptic splice sites that redefine intron boundaries and consequently frameshifts the mature transcript (Reddy et al., 2007). This is consistent with our cDNA clone-seq analysis identifying a type 3 splice variant of *ncbp-1* from line *ncbp-1/2* #8. However, the type 2 *ncbp-1* variant of *ncbp-1/2* #2 does not appear to be the result of splice site disruptions. Furthermore, it codes for full length nCBP-1 with a 12 amino acid extension. It is possible that similar unpredicted splice variants exist at low abundance in *ncbp-1/2* #8. Complementation assays will need to be performed to determine whether such putative splice variants can be utilized by the viruses. The level of these transcripts and/or their encoded protein's affinity for CBSV and UCBSV VPgs are likely low considering the clear impact on CBSD development. Second, CBSV and UCBSV VPgs may have some inherent, low-level affinity for the other eIF4E isoforms. Co-expression of the cassava eIF(iso)4E-1 and -2 with VPg from both species showed weak reporter activation that could be interpreted as weak interaction or reporter auto-activation as seen in the TuMV VPg plus empty vector control (Fig. 2). VPg is an intrinsically disordered protein, which could enable it to bind several different proteins (Jiang and Laliberté, 2011). The ability to use multiple eIF4E isoforms has precedence, such as in *Pepper veinal mottle potyvirus* for which simultaneous mutations of both eIF4E and eIF(iso)4E is required to restrict infection (Gauffier *et al.*, 2016; Ruffel *et al.*, 2006). Recruitment of eIF4E or the two eIF(iso)4E isoforms by CBSV/UCBSV could result in sub-optimal viral replication or movement, resulting in lower symptom severity and incidence. This has previously been hypothesized by Chandrasekaran *et al*. (2016) for breaking of *eif4e*-mediated resistance in cucumber. Further investigation will be required to test this hypothesis in cassava.

CBSD remains a major threat to food security in sub-Saharan Africa. Mitigation of crop losses is imperative to sustaining Africa's rapidly growing population. Due to the challenges of

breeding cassava, genetic editing strategies provide an attractive means to engineer disease resistance. In this study, we show that simultaneous CRISPR/Cas9-mediated editing of the *nCBP-1* and *nCBP-2* genes confers statistically significantly elevated resistance to CBSD. Editing of these host translation factors significantly hampers CBSV accumulation in the plant. By stacking this approach with other forms of resistance such as RNAi, potential exists to provide improved cassava varieties with robust and durable resistance to CBSD.

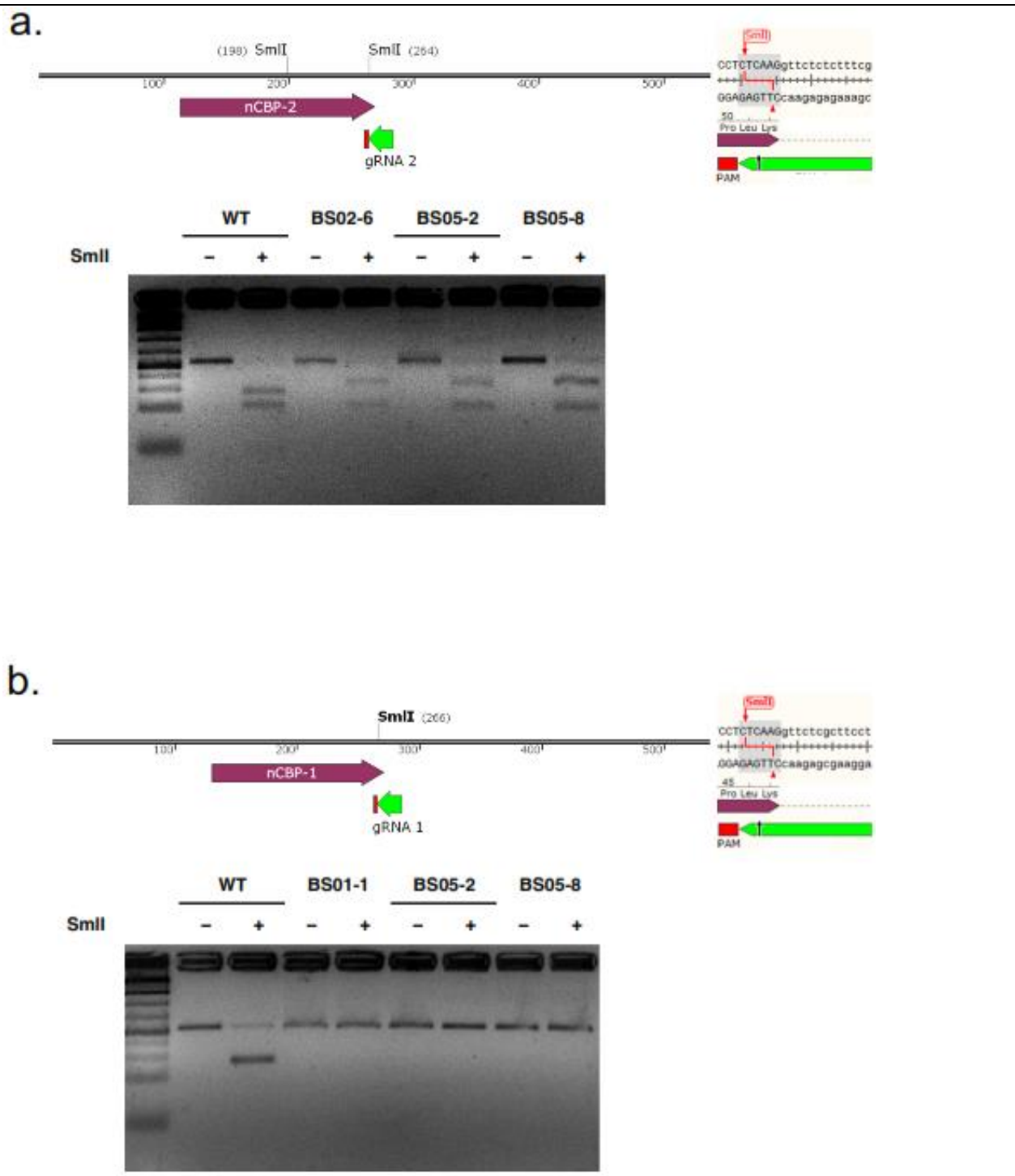


Figure S3-1. CRISPR/Cas9-induced mutagenesis evident in *nCBP-1* (a) and *nCBP-2* (b) via restriction enzyme site loss (RESL). PCR amplicons of targeted regions were digested with *Sml*I. Map of amplicons with nCBP exon (purple), protospacer adjacent motif (red), gRNA spacer (green), predicted Cas9 cut site (black arrow), and overlapping *Sml*I restriction enzyme recognition site (bold, red). Bands are measured relative to O'Gene Ruler 1 kb Plus Ladder. Experimental banding pattern is consistent with predicted RESL.

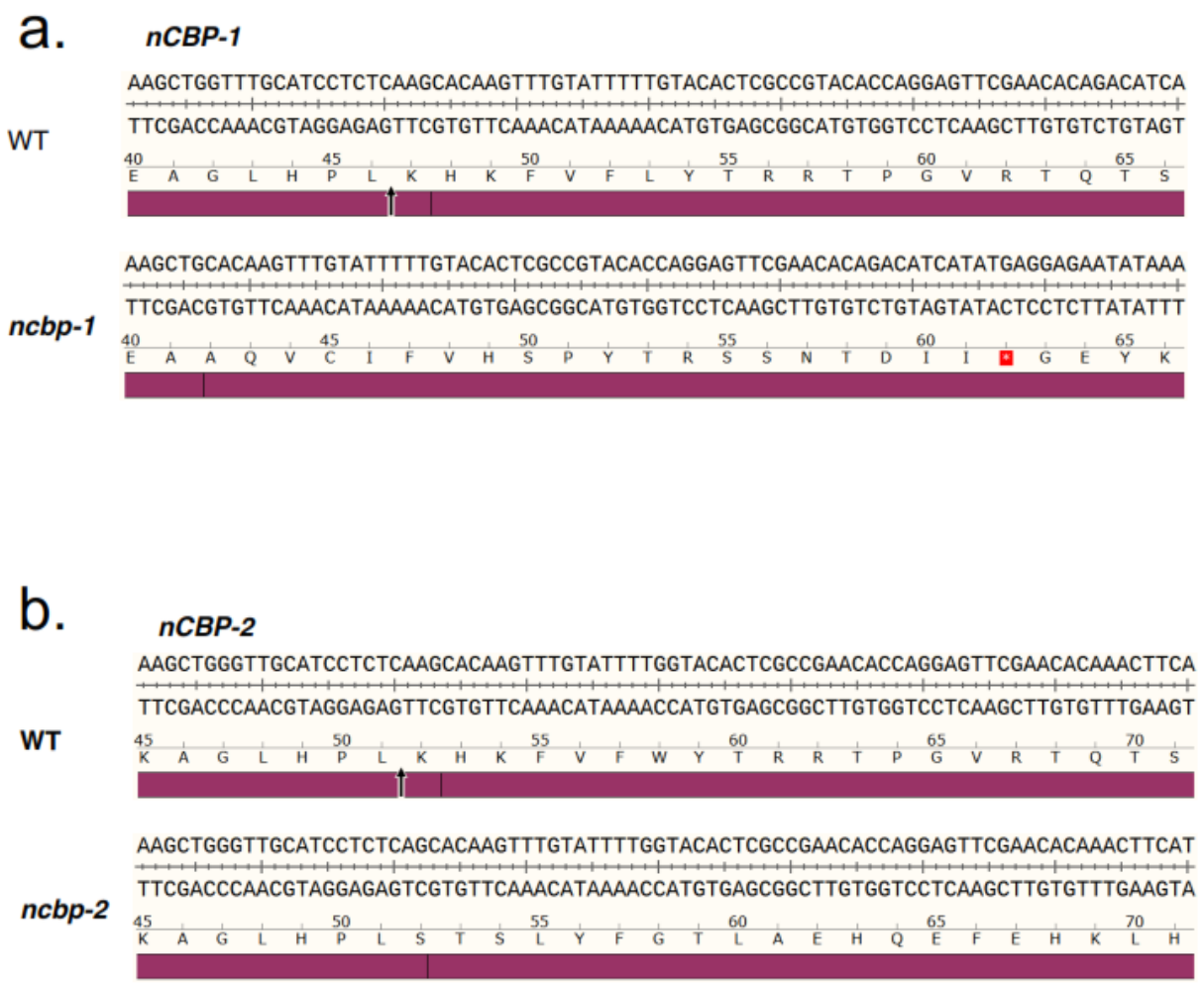


Figure S3-2. CRISPR/Cas9 –induced mutagenesis creates out of frame alternate splice variants. Exon 1 and exon 2 splice junction of nCBP-1 (a) and nCBP-2 (b) were examined via sequence analysis of cDNA. Predicted Cas9 cut site is shown as a black arrow. STOP codon is shown as starred, red box.

Target Gene	Construct	Line	Target Position	Mutation Zygosity	Genotype	Effect
nCBP-1	BS01	1	133	Homozygous	d3	Frameshift
		3	133	Chimeric	d4, i1, d1, d2	Frameshift, Frameshift, Frameshift, Frameshift
		4	133	Bi-allelic	d3, d9	Frameshift, Frameshift
		5	133	WT	WT	No effect
		6	133	Homozygous	i1	Frameshift
		7	133	Chimeric	d4, d1, d19, d34i16	Frameshift, Frameshift, Frameshift, Frameshift
	BS03	2	2677	WT	WT	No effect
		3	2677	Homozygous	d9	Frameshift
		4	2677	Bi-allelic	i3d2, i1	Frameshift, No effect
		5	2677	Homozygous	i1	No effect
		6	2677	Homozygous	d1	Frameshift
		7	2677	Bi-allelic	d3, i1	1 AA deleted and 1 AA changed, No effect
		8	2677	Bi-allelic	d12, i1	Frameshift, No effect

		9	2677	Homozygous	i1	No effect
		10	2677	Bi-allelic	d93i2, d2	Frameshift, Frameshift
		11	2677	Bi-allelic	d5, d1	Frameshift, Frameshift
nCBP-2	BS02	1	148	Chimeric	d3, d6, d7, i2d8	1 AA deletion and 1 AA changed, Frameshift, Frameshift, Frameshift
		2	148	Bi-allelic	d5, d2	Frameshift, Frameshift
		4	148	Bi-allelic	i4d127, i8d20	Frameshift, Frameshift
		5	148	Chimeric	d17, d52, d1, i2d4	Frameshift, Frameshift, Frameshift, Frameshift
		6	148	Homozygous	d1	Frameshift
		7	148	Bi-allelic	d1, d17	Frameshift, Frameshift
	BS04	1	2763	Heterozygous	WT, i1	No effect , Frameshift
		2	2763	Bi-allelic	i1, d7	Frameshift, Frameshift
		3	2763	Chimeric	i1, d7, d4	Frameshift, Frameshift, Frameshift
		4	2763	Bi-allelic	d4, i1	Frameshift, Frameshift
		5	2763	Bi-allelic	d2, d6	Frameshift, 2 AA deletion
		6	2763	WT	WT	No effect
		8	2763	Homozygous	i1	Frameshift
		9	2763	Bi-allelic	d2, d7	Frameshift, Frameshift
		10	2763	Bi-allelic	i1d8, d2	Frameshift, Frameshift
		11	2763	Bi-allelic	d2, i1d10	Frameshift, Frameshift
		12	2763	Bi-allelic	d5, d11	Frameshift, Frameshift
		13	2763	Homozygous	d2	Frameshift
		14	2763	Bi-allelic	d7, d2	Frameshift, Frameshift
		15	2763	Bi-allelic	i1, d2	Frameshift, Frameshift
		16	2763	Bi-allelic	i1, d5	Frameshift, Frameshift
nCBP-1/2	BS05	1	148	Bi-allelic	d7, d1	Frameshift, Frameshift
			133	Bi-allelic	d2, d4	Frameshift, Frameshift
		2	148	Homozygous	d11	Frameshift
			133	Homozygous	i1	Frameshift
		3	148	Bi-allelic	i1, d1	Frameshift, Frameshift
			133	Bi-allelic	d1, d5	Frameshift, Frameshift
		4	148	Bi-allelic	d5, d4	Frameshift, Frameshift
			133	Bi-allelic	d2, d1	Frameshift, Frameshift
		5	148	Bi-allelic	i1, d6	Frameshift, Frameshift
			133	Bi-allelic	d4, d9	Frameshift, Frameshift
		6	148	WT	WT	No effect
			133	WT	WT	No effect
		7	148	Bi-allelic	i1, d15	Frameshift, Frameshift
			133	Bi-allelic	d3, d5	Frameshift, Frameshift
		8	148	Homozygous	d1	Frameshift
			133	Bi-allelic	d3, d1	Frameshift, Frameshift
eIF4E	BS06	3	1892	Bi-allelic	d1, d7	Frameshift, Frameshift
		4	1892	Homozygous	i1	Frameshift
		5	1892	Bi-allelic	d3, d4	1 AA deletion, Frameshift
		6	1892	Bi-allelic	d3, d4	1 AA deletion, Frameshift
		7	1892	Homozygous	d2	Frameshift
		8	1892	Homozygous	i1	Frameshift
		11	1892	Bi-allelic	d3, d12	1 AA deletion, 4 AA deletion
	BS07	1	-16	WT	WT	No effect
		2	-16	Bi-allelic	d2i1, d15i12	Start codon removal, Start codon removal
		9	-16	Bi-allelic	d4, i1	No effect

Table S3-1. Genotypes of all transgenic T0 cassava lines. WT, wild-type alleles; bi-allelic, two different mutated alleles; heterozygous, wild-type and mutated alleles; chimeric, more than two mutated alleles. d# and i# refer to deletions and insertions, respectively, with the number of bases mutated denoted by #. Highlighted transgenic events were used in CBSV/UCBSV assays.

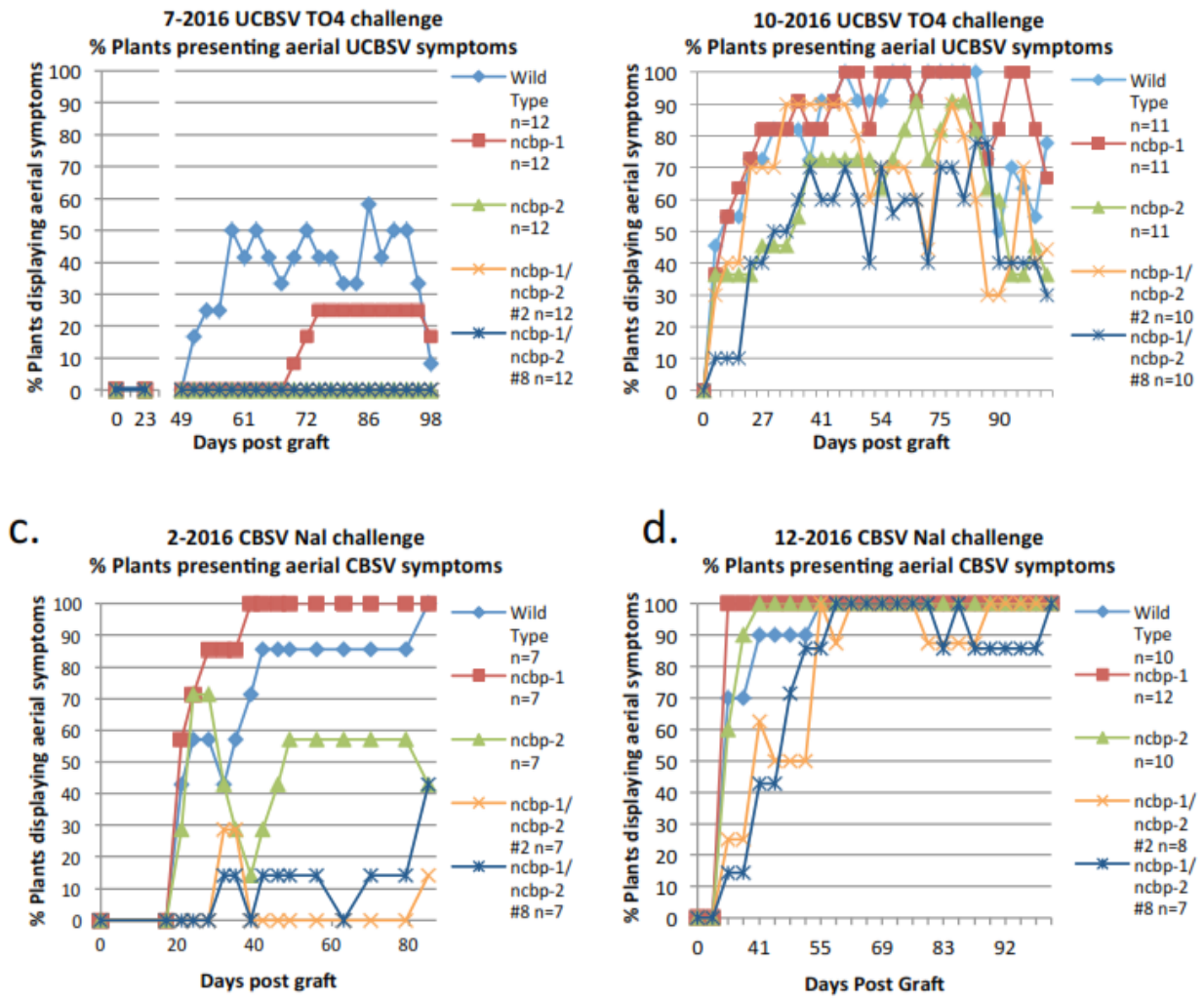


Figure S3-3. *ncbp-1/ncbp-2* double mutants exhibit reduced UCBSV symptom incidence and delayed CBSV symptom onset. (a), (b), aerial symptom incidence reported as percent of wild type, *ncbp-1*, *ncbp-2*, or *ncbp-1/ncbp-2* plants bud-graft inoculated with UCBSV T04 (n≥10). (c), (d), aerial symptom incidence as previously described in plants inoculated with CBSV Naliendele (n≥7).

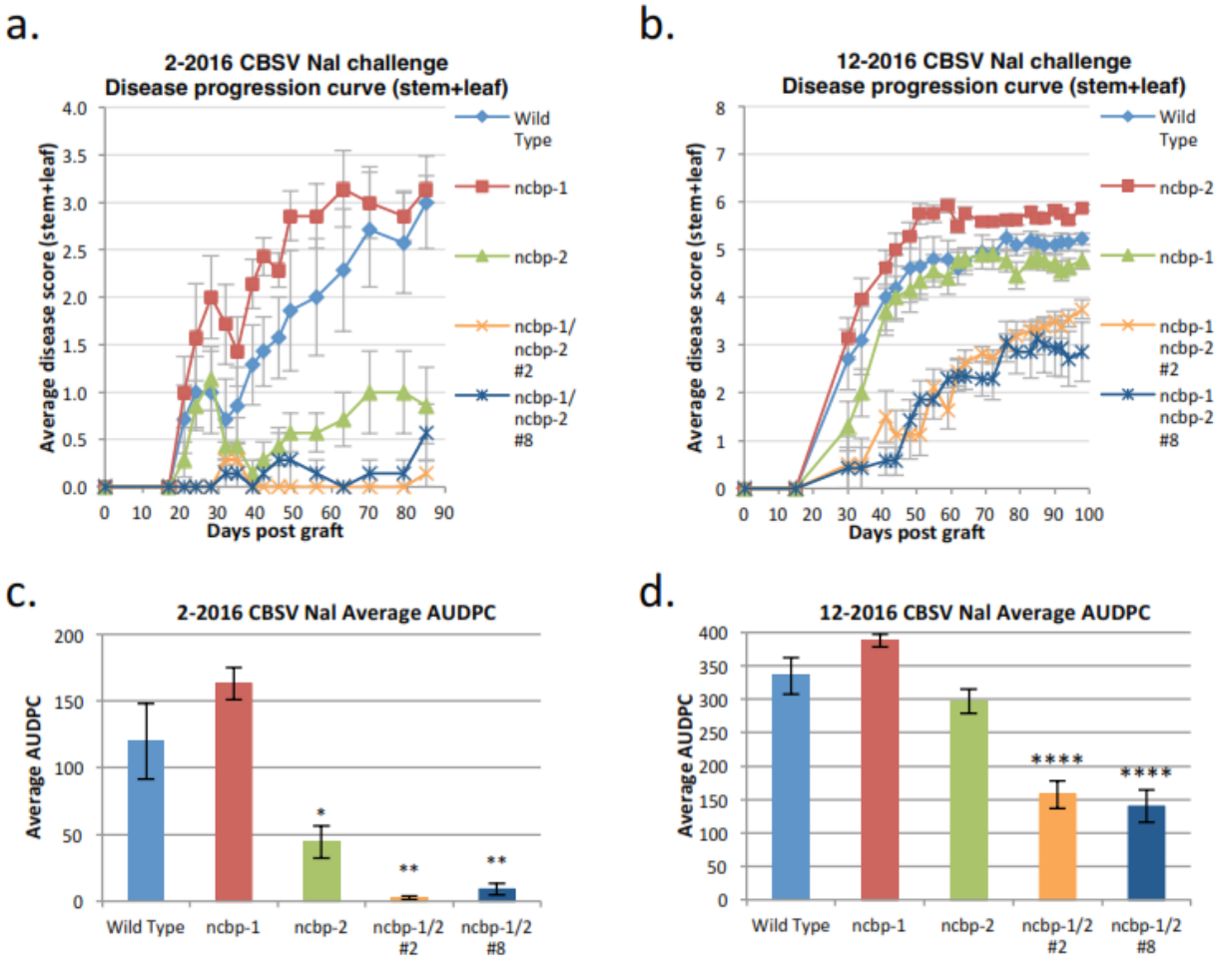


Figure S3-4. *ncbp-1/ncbp-2* double mutants exhibit reduced aerial CBSV symptom severity. (a), (b), disease progression curves of wild type, *ncbp-1*, *ncbp-2*, or *ncbp-1/ncbp-2* plants bud-graft inoculated with CBSV Naliendele (n≥7). Leaf and stem symptoms were each scored on a 0-4 scale and summed to obtain an aggregate aerial score. (c), (d), average area under the disease progression curve (AUDPC) derived from data plotted in A and B. Error bars indicate standard error of the mean. Statistical differences were detected by Welch's t-test, α=0.05, *≤0.05, **≤0.01, ****≤0.0001.

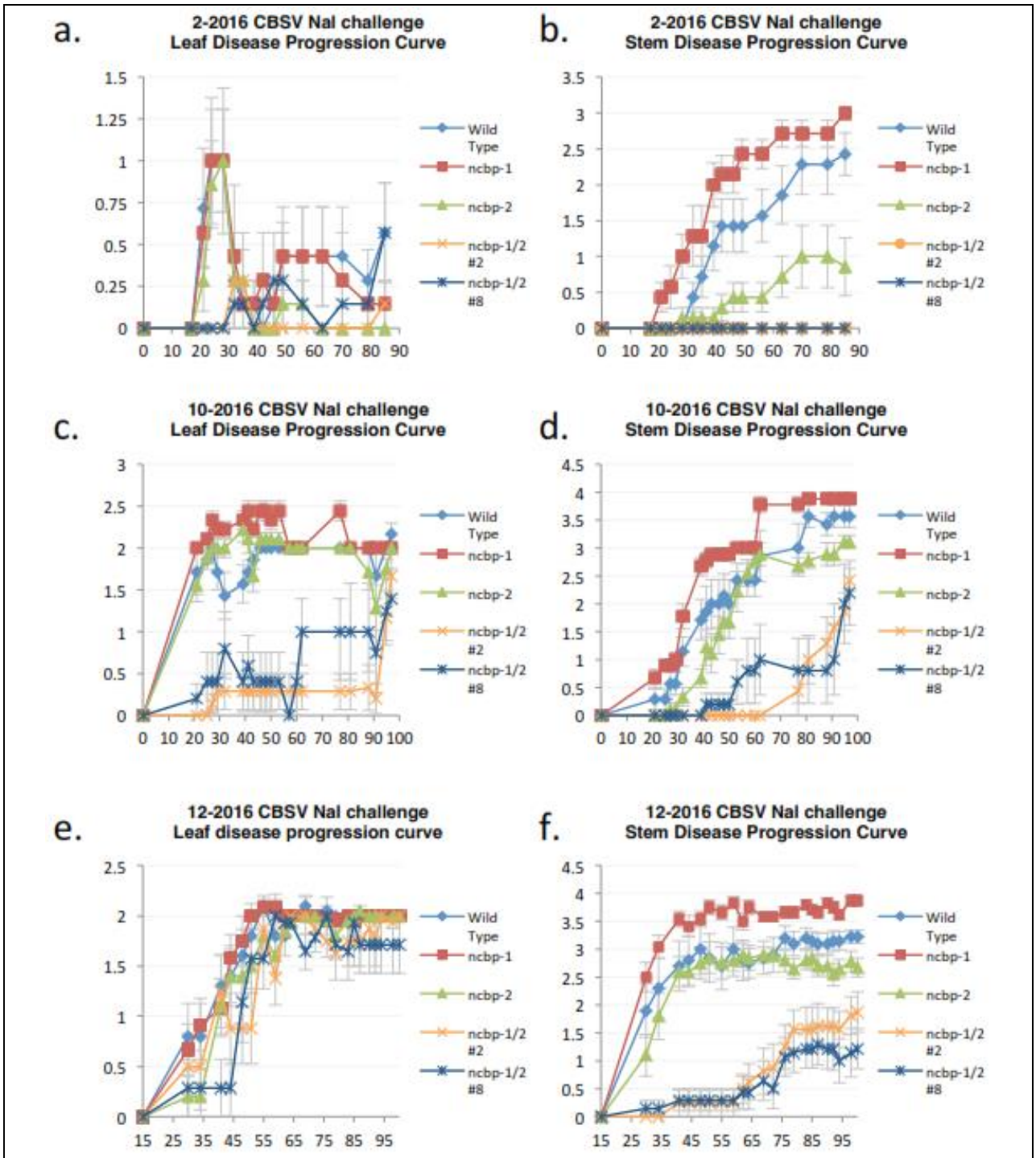


Figure S3-5. *ncbp-1/ncbp-2* double mutant stem symptom severity is consistently reduced across all experiments. Separate leaf and stem disease progression curves for wild type, *ncbp-1*, *ncbp-2*, or *ncbp-1/ncbp-2* plants bud-graft inoculated with CBSV Naliendele (n≥7). Leaf and stem symptoms were each scored on a 0-4 scale. (a), (c), and (e) represent leaf disease progression curves from three different experiments while (b), (d), and (f) represent corresponding stem disease progression curves. Error bars represent standard error of the mean.

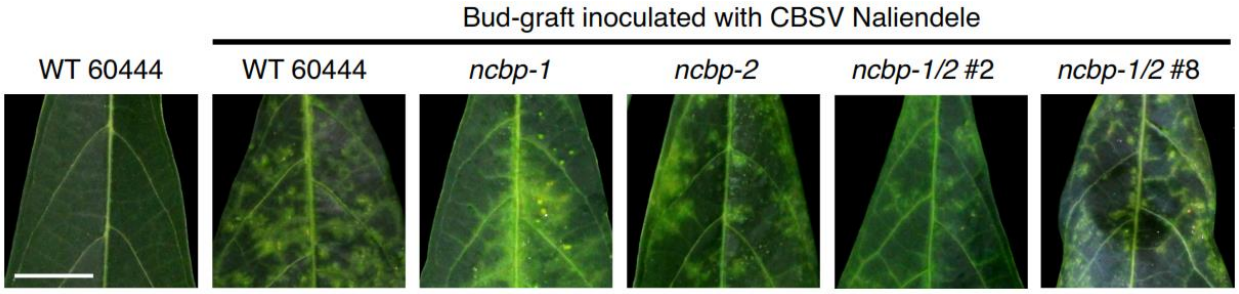


Figure S3-6. 12-2016 CBSV challenge leaf symptom severity is similar across all genotypes. Wild type, *ncbp-1*, *ncbp-2*, or *ncbp-1/2* plants bud-graft inoculated with CBSV Naliendele isolate all develop widespread chlorotic leaf symptoms. Leaf images were taken near 12-2016 challenge endpoint. Scale bar denotes 1 cm.

Tissue specific expression of cassava *eIF4E* isoforms

Gene	FEC	Fibrous Root	Lateral Bud	Leaf	Mid Vein	OES	Petiole	RAM	SAM	Stem	Storage root
eIF(iso)4E-1	51	28	19	15	12	42	20	70	36	26	28
eIF(iso)4E-2	85	65	49	72	48	81	67	148	74	73	73
nCBP-2	154	185	362	96	113	597	105	142	165	121	1259
nCBP-1	15	51	23	117	91	11	121	38	18	71	114
eIF4E	72	94	74	69	61	72	82	210	104	89	87

shiny.danforthcenter.org/cassava_atlas/

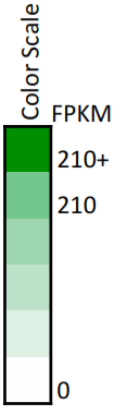


Figure S3-7. *nCBP-2* is highly expressed in storage roots. Heat map describing tissue specific expression of cassava *eIF4E* isoforms. Data was extracted from the Bart Lab Cassava Atlas (http://shiny.danforthcenter.org/cassava_atlas/). Expression values are defined as fragments per kilobase of transcript per million mapped reads (FPKM).

4. CRISPR/Cas9-mediated viral interference of *Tomato yellow leaf curl virus* in tomato.

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Background

The *Geminiviridae* family consists of monopartite or bipartite single-stranded DNA viruses. This family acquires its name from its unique geminated icosahedral capsid structure. Together with the previously described potyviruses, they cause severe harm to production of important crop plants, including cassava, maize, cotton, and tomato (Hohn *et al.*, 2009). The whitefly species *Bemisia tabaci* that spreads *Cassava brown streak virus* is also responsible for transmitting geminiviruses (Cohen and Harpaz, 1964). Following delivery, geminiviruses can cause severe symptoms, including chlorosis, leaf curling and stunting, especially when infecting a plant in concert with other viruses (Mansoor *et al.*, 2006). The geminivirus *Tomato yellow leaf curl virus* (TYLCV) is particularly problematic following its global spread and threat to tomato production in temperate parts of the world (Lefeuvre *et al.*, 2010; Mansoor *et al.*, 2006). High levels of geminivirus genetic variability arise due to high rates mutation and recombination (Duffy and Holmes, 2008; Jeske *et al*., 2001). As a result of rapid evolution and improved lab diagnostics, the number of discovered *Geminiviridae* species has grown rapidly (Rosario *et al.*, 2012). In addition, large shifts or invasions by the insect vector population, as has been observed by whiteflies in China, can introduce geminiviruses to new viral partners with which they may recombine and add to their genetic variability (Pan *et al.*, 2011; Pan *et al*., 2012).

Strategies to control geminivirus spread include insecticides, RNA interference, zinc finger nuclease targeting, and peptide aptamers that interfere with replicase activity. However, these strategies can have some limitations in their use. Insecticide sprays run the risk of selecting for whiteflies that are resistant to the chemicals, as may be the case for biotype Q whitefly in China (Inoue-Nagata *et al.*, 2016). In addition, insecticides may not be affordable or accessible to small-holder farmers (Alene *et al.*, 2005). Breeding programs have sought to introgress resistance or tolerance found in some wild tomato species into the domesticated tomato. However, the nature of this resistance or tolerance may be multigenic, complicating breeding approaches (Pico *et al.*, 1996). More recent control strategies that apply RNA interference and zinc finger nucleases that target the geminivirus have broken down in field trials and may have limited range and durability due to sequence specificity in the face of a diverse and rapidly evolving virus family (Fuentes *et al*., 2016; Chen *et al.*, 2014). In contrast, peptide aptamers can impact a diversity of geminiviruses as they bind the Rep protein and interfere with its function. However, virus accumulation was only delayed (Reyes *et al.*, 2013). More robust resistance may be acquired by stacking this strategy with other forms of geminivirus control.

The CRISPR/Cas9 system has emerged as a powerful tool for genome editing in various plants and animals. This system's original role as a form of molecular immunity against phages

may also be extended to plants as a form of immunity against DNA viruses. CRISPR/Cas9-mediated viral interference in tomato may serve as an additional form of resistance to TYLCV infection. Normal TYLCV replication within the host cell may by disrupted by Cas9-mediated cutting of the virus genomic DNA (Figure 4-1). The feasibility of this approach has already been demonstrated against TYLCV, *Beet curly top virus* (BCTV), and MeMV in *A. thaliana* and *N. benthamiana* (Ali *et al.*, 2015; Ali *et al.*, 2016). In these studies, CRISPR/Cas9 was stably transformed into plants and led to reduced viral DNA accumulation and symptom development in the transgenic plants. Extension of this strategy to tomatoes could have a global impact, as TYLCV is also spread globally and hampering food production and security. An added advantage of the CRISPR system is the ability to employ multiple sgRNA in the same CRISPR/Cas9 expression cassette for multiplex targeting (Cong *et al.*, 2013). Due to the high levels of genetic variability and rate of mutation, selecting a single Cas9-target site may be insufficient for broad and durable resistance. Design and deployment of multiple sgRNAs to multiple conserved regions of the genome and to different virus species will offer greater protection to the tomato crop.

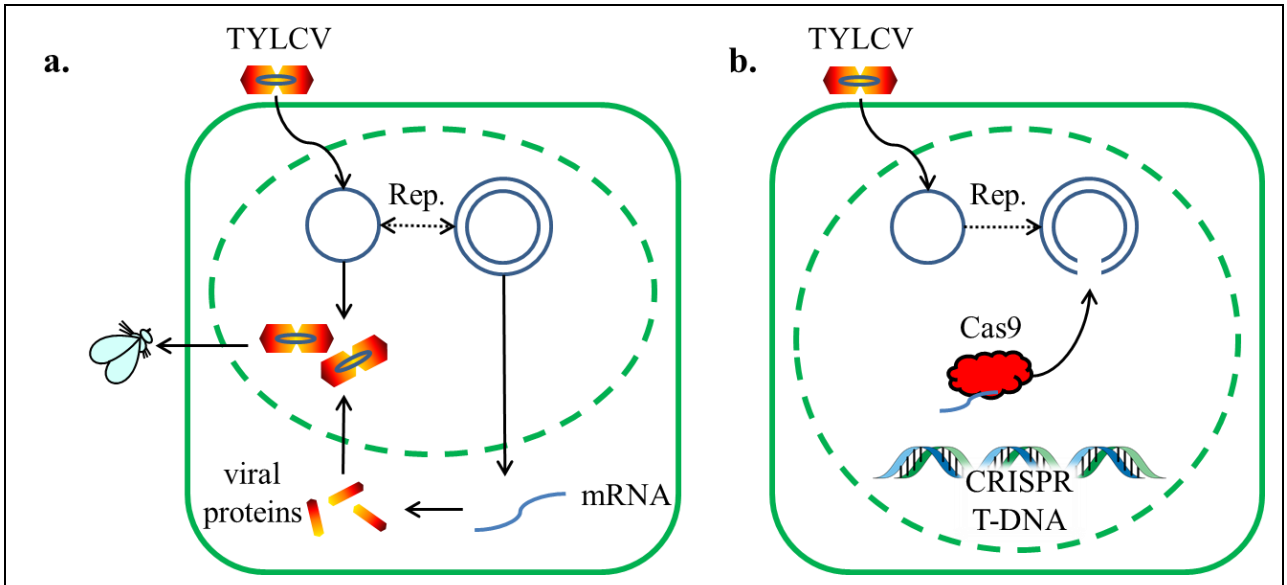

Figure 4-1. TYLCV lifecycle and strategy for CRISPR-mediated viral interference. (a) Infection of plant cell by TYLCV releases single stranded, ss, DNA genome. ssDNA is replicated in double stranded, ds, DNA and ssDNA forms. Expression of viral genes yields viral proteins essential for virion assembly with its ssDNA genome. Virions are transmissible via acquisition by an insect vector. (b) Expression of CRISPR/Cas9 transgene yields active Cas9/gRNA complex that is directed to dsDNA TYLCV genome. Cas9-mediated cutting of TYLCV genome interferes with virus life cycle.

Results

Identification of CRISPR/Cas9 targets conserved across multiple TYLCV isolates

To apply the CRISPR/Cas9 system for reducing TYLCV replication in tomato, we designed sgRNAs to target the viral genomes of sequenced TYLCV isolates on the National Center for Biotechnology Information database (NCBI, 1988). Isolate accession numbers are shown in Table 4-1. We first sought to reduce the risk of genome restoration by avoiding sgRNA that were complementary to regions with high frequencies of recombination (Figure S4-1). Recombination with another TYLCV isolate or species that removes the intended Cas9 target would undermine efforts to suppress virus replication. Next, we used the parameter of 5'-G-N_{19}-NGG-3' to search for conserved Cas9 targets across the available TYLCV sequences. We selected a 5' G to facilitate sgRNA expression from an *A. thaliana U6* promoter. We found two positions in the mapped TYLCV genome, position (pos)1874 and pos1936, that were conserved across 11/12 and 10/12 isolates, respectively, from around the world (Table 4-1). Despite virus#11 pos1936 does not match the other examined isolates, its pos1874 is broadly conserved. Likewise, viruses #4 and 5 pos1874 are not broadly conserved, but pos1936 matches the majority of other sequences. Therefore, simultaneous deployment of dual sgRNAs complementary to both of these positions provides broader TYLCV isolate targeting than any single sgRNA. Pos1936 and pos1874 overlap the replication-associated protein, Rep (Figure 4-2a). Complementary sgRNA in complex with Cas9 would target the virus's essential replication function. The predicted Cas9 cut sites of these sgRNA are 62 bp apart (Figure 4-2b). If both positions are targeted simultaneously, a portion of this critical open reading frame (ORF) would be excised. In review, we designed sgRNA that avoided regions of high recombination frequency, were broadly conserved across sequenced TYLCV isolates, and targeted a critical ORF for TYLCV survival.

Virus		Rep(+) pos. 1874	Rep(+) pos. 1936
1	TYLCV2 (NCSU)	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
2	KC428753 TYLCV isolate KSQ1-3 Israel	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
3	AB110218 TYLCV Israel	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
4	AB116631 TYLCV Israel	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTTTGGG
5	NC_000869 TYLCV Thailand	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTTTGGG
6	AF105975 TYLCV Portugal	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
7	KC428753 TYLCV China	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
8	LOCUS JX444575 Jordan	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
9	KC312655 TYLCV China isolate AH-BB	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
10	HW046992 TYLCV	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
11	HE819243 TYLCV Oman	GGATTGCAGAGGAATATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG
12	JQ928347 TYLCV Iran	GGATTGCAGAGGAAGATAGTGGG	GTTGCTTTGCCAGTCCCTCTGGG

Table 4-1. CRISPR/Cas9 targets are conserved across multiple TYLCV isolates. Mismatches shown in red. Protospacer adjacent motif (PAM) is underlined.

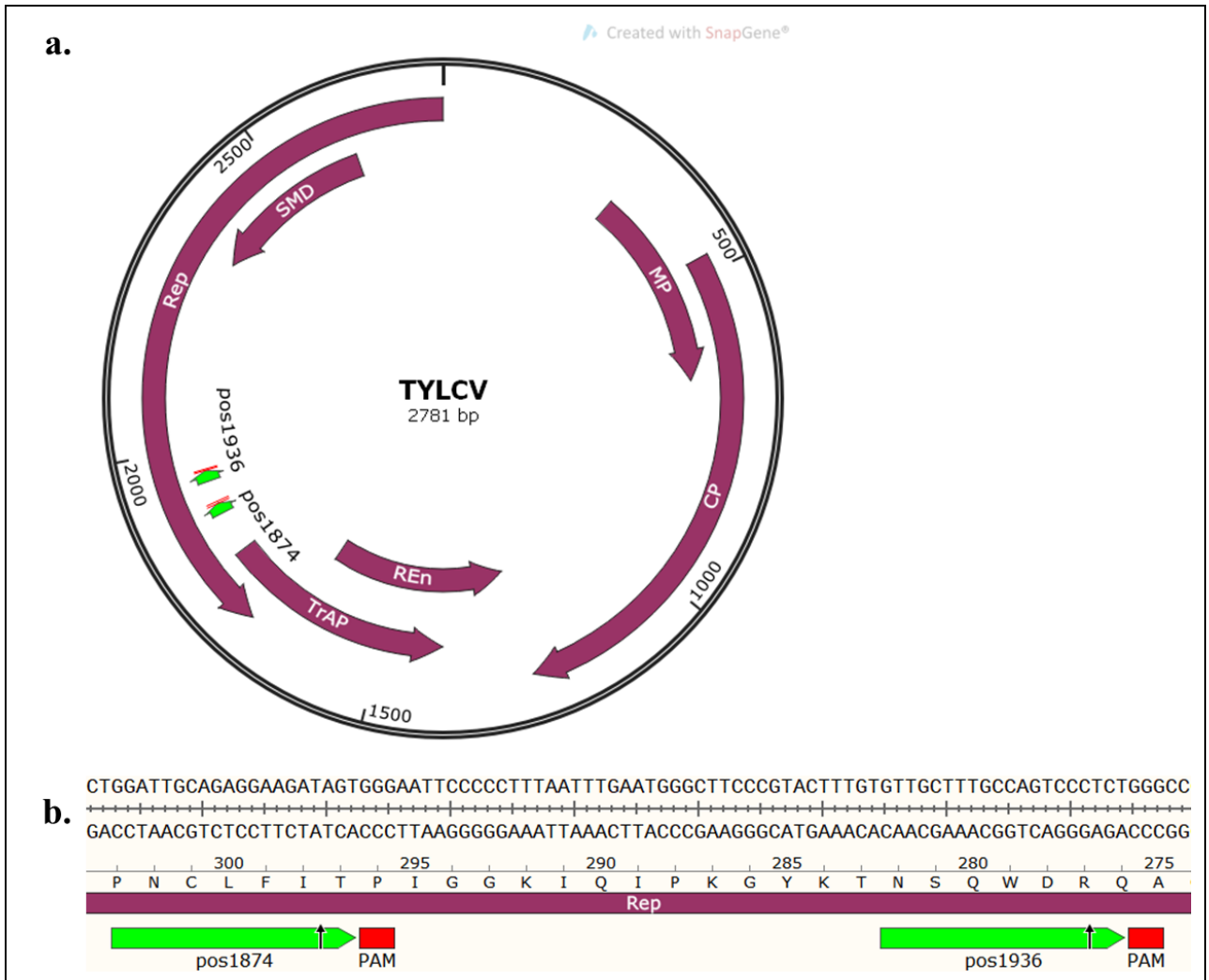

Figure 4-2. CRISPR/Cas9 targets overlap replication-associated protein ORF. Features annotated in purple. CP, coat protein; MP, movement protein; Rep, replication-associated protein; TrAP, transcriptional activator protein; REn, replication enhancer protein; SMD, symptom and movement determinant. sgRNA spacers annotated in green. Protospacer adjacent motifs, PAMs, annotated in red. Predicted Cas9 cut site shown by black arrow.

Transient CRISPR/Cas9 targeting of TYLCV DNA on surrogate gemini-vector

We assembled the selected sgRNA into CRISPR/Cas9 plant expression vectors to function individually (Figures S4-3, S4-4, S4-5, S4-6) and simultaneously (Figures S4-7, S4-8). Expression of each sgRNA is driven by an *A. thaliana U6* promoter and terminated at the end of the scaffold sequence by a poly-T sequence. Prior to transformation of these pos1936- and pos1874-targeting CRISPR/Cas9 expression vectors into tomato, we sought to test their activity via a transient CRISPR/Cas9 *in planta* assay (Figure S4-2). The TYLCV pos1936 and pos1874 target region was PCR amplified as a ~500 bp fragment and cloned into a surrogate gemini-vector (Figures S4-9, S4-10). This vector is a derivative of the pLSLZ.D.R vector from which the essential components for replicon synthesis were cloned into a minimal backbone from the pEAQ-HT plasmid (constructed by Alex Schultink; Baltes *et al.*, 2014; Sainsbury *et al.*, 2009). This gemini-vector facilitates delivery and amplification of DNA fragments of interest *in planta*. Briefly, *Agrobacterium* strains carrying a CRISPR/Cas9 expression vector and cloned gemini-vector were co-infiltrated into *N. benthamiana*. The respective T-DNAs of these constructs may be integrated into the plant genome. Subsequently, the gemini-vector T-DNA releases and replicates DNA replicons bearing the TYLCV target region. CRISPR T-DNA expression yields Cas9/sgRNA complexes that are specific for the pos1936 and/or pos1874 target sites. Cas9 may target the sgRNA-matching replicons or the gemini-vector T-DNA itself. Following Cas9-induced mutagenesis of the gemini-vector T-DNA, newly released replicons will carry the pos1936 and/or pos1874 mutation(s) at the target site(s). This gemini-vector strategy serves to amplify the concentration of mutagenized TYLCV fragment DNA to levels easily detectable by PCR and Sanger sequencing.

DNA was extracted from *N. benthamiana* leaves that were co-infiltrated with the TYLCV-bearing gemini-vector and a no-sgRNA construct, pos1874-targeting construct, pos1936-targeting construct, and dual-targeting construct. Gel electrophoresis of PCR amplicons shows a smaller fragment from the dual-targeting construct as compared to the no-sgRNA control (Figure 4-3a). The size of this fragment is consistent with a 62 base pair excision of the DNA between the pos1874 and pos1936 predicted cut sites (Figure 4-2). To verify the sgRNA functioned individually, PCR amplicons from the individual site-targeting constructs were analyzed via Sanger sequencing. Cas9-induced mutagenesis can create a wide array of mutations that result in a mixed signal and indeterminate sequence read. Analysis of the pos1874 and pos1936 sequences shows this indeterminate sequence read starting at the predicted cut sites (Figure 4-3b, c). When this analysis was applied to PCR amplicons from the dual site-targeting construct, indeterminate sequence reads also followed the predicted Cas9 cut sites (Figure 4-3d). The observed mutagenesis of the TYLCV DNA fragment on the gemini-vector indicate that the assembled CRISPR constructs are functional and ready for delivery into tomato.

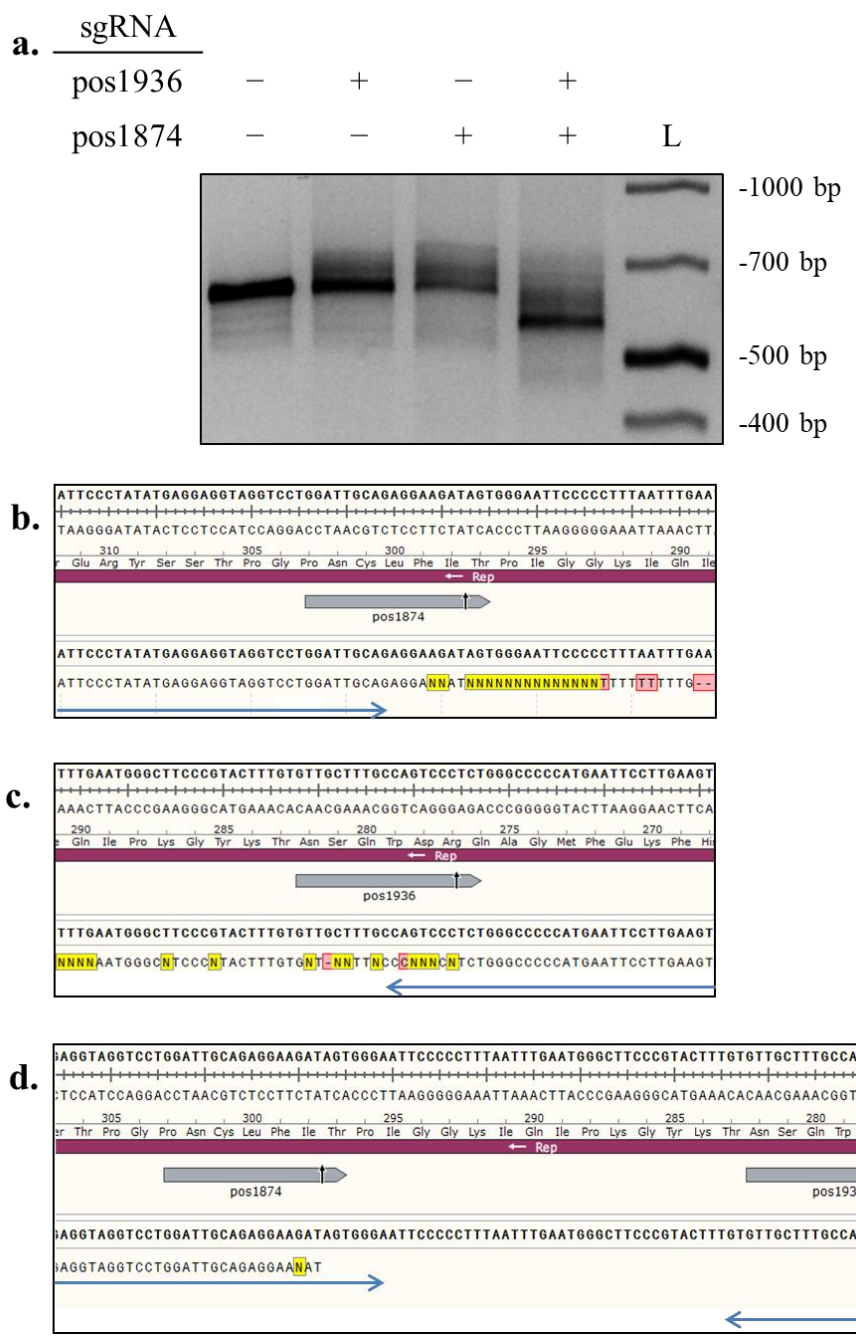

Figure 4-3. Transient CRISPR/Cas9 *in planta* assay shows TYLCV-targeting sgRNAs are functional. (a) PCR amplicons of transient assay DNA extract run on high % TAE gel. Co-delivery of both sgRNAs yields smaller TYLCV PCR amplicon. (b) Sequence analysis of PCR product from pos1874-only transient assay. Sequence read direction shown by blue arrow. TYLCV Rep feature annotated in purple. sgRNA spacers annotated in gray. Predicted Cas9 cut site shown by black arrow. (c) Sequence analysis of PCR product from pos1936-only transient assay. (d) Sequence analysis of PCR product from co-delivered pos1874 and pos1936 transient assay. Sequence reads from opposite directions. Highly mixed reads result in sequence alignment termination.

CRISPR/Cas9 transgenic tomato lines exhibit reduced TYLCV symptom severity and viral DNA presence

The pos1936-, pos1874-, and dual-targeting constructs were transformed into the FL8000 background to produce transgenic lines named MG1, MG2, and MG3, respectively (Figure 4-4a). Transgenic lines were selected on agar supplemented with kanamycin and verified to carry the CRISPR/Cas9 expression T-DNA via PCR screening. Seeds from this T0 generation were harvested and potted in soil. Five to fifteen plants from wild-type and transgenic lines were grown for TYLCV challenge. An infectious clone of TYLCV was delivered into two-week-old T1 tomato plants via *Agrobacterium*-mediated inoculation. Approximately 4 weeks after infection, these plants were scored for TYLCV symptom development ranging from no symptoms, 1, to severe chlorosis and stunting, 4 (Figure S4-11). 26 days after infection, lines MG1-9 and MG1-14 collectively showed statistically significant reduction in symptom score as compared to the wild-type (Figure 4-4b). Line MG1-1 showed severe chlorosis and stunting as compared to the MG1-9 (Figure 4-5). 24 and 28 days after infection, MG3-12 and MG3-6 lines, respectively, showed statistically significant reduction in symptom scores, p-value ≤0.0001 (Figure 4-4c, d). Plants from several transgenic lines collectively scored wild-type or intermediate TYLCV symptom development.

a.

Transgenic CRISPR lines	pos1936 sgRNA	pos1874 sgRNA
MG1	+	–
MG2	–	+
MG3	+	+

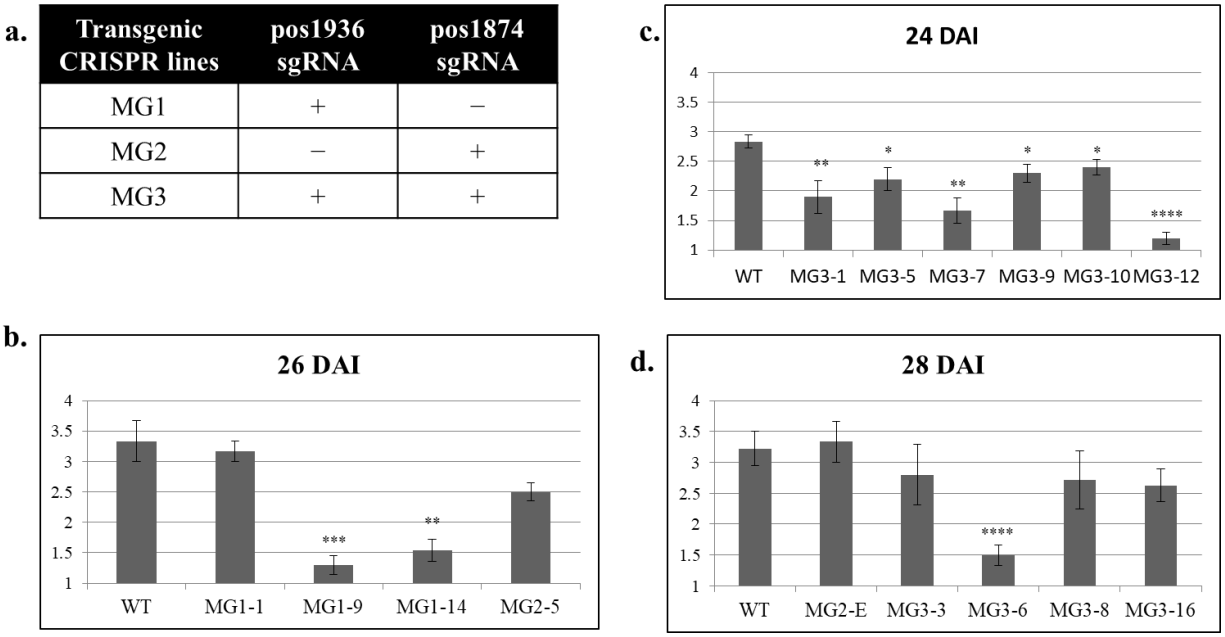

Figure 4-4. TYLCV challenge symptom scoring of segregating transgenic CRISPR/Cas9 lines shows significantly reduced symptoms. (a) Naming scheme for transgenic CRISPR lines bearing pos1936 and pos1874 sgRNA individually and simultaneously. (b) TYLCV infection symptom scores of first challenge trial 26 days after infection, DAI. Statistical significance was detected by Welch's t-test, n≥6, α=0.05, *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001. (c) TYLCV infection symptom scores of second challenge trial 24 DAI. Statistical significance was detected by Welch's t-test, n≥6, α=0.05. (d) TYLCV infection symptom scores of third challenge trial 28 DAI. Statistical significance was detected by Welch's t-test, n≥5, α=0.05, ****≤0.0001.

Figure 4-5. TYLCV symptom development in susceptible and resistant transgenic plants 26 days after infection. MG1-1 exhibits severe chlorosis and stunting. MG1-9 shows no symptoms.

To examine whether the presence of Cas9 was associated with TYLCV symptom development and virus accumulation, plants from transgenic lines were assessed for *Cas9* and TYLCV DNA via PCR. Consistent with symptom scoring, symptomatic wild-type and MG1-1 lines showed TYLCV DNA as Cas9 DNA was not detected (Figure 4-6a). In contrast, line MG1-9 plants, which collectively showed a significant reduction in symptom score carried the *CRISPR/Cas9* T-DNA and TYLCV were not detected. Likewise for MG3 transgenic lines in which both pos1874 and pos1936 were targeted, symptomatic MG3-10 plants presented TYLCV DNA but no Cas9 (Figure 4-6b). In the case of MG3-12, TYLCV DNA was absent except for two samples that were also positive for the Cas9 transgene.

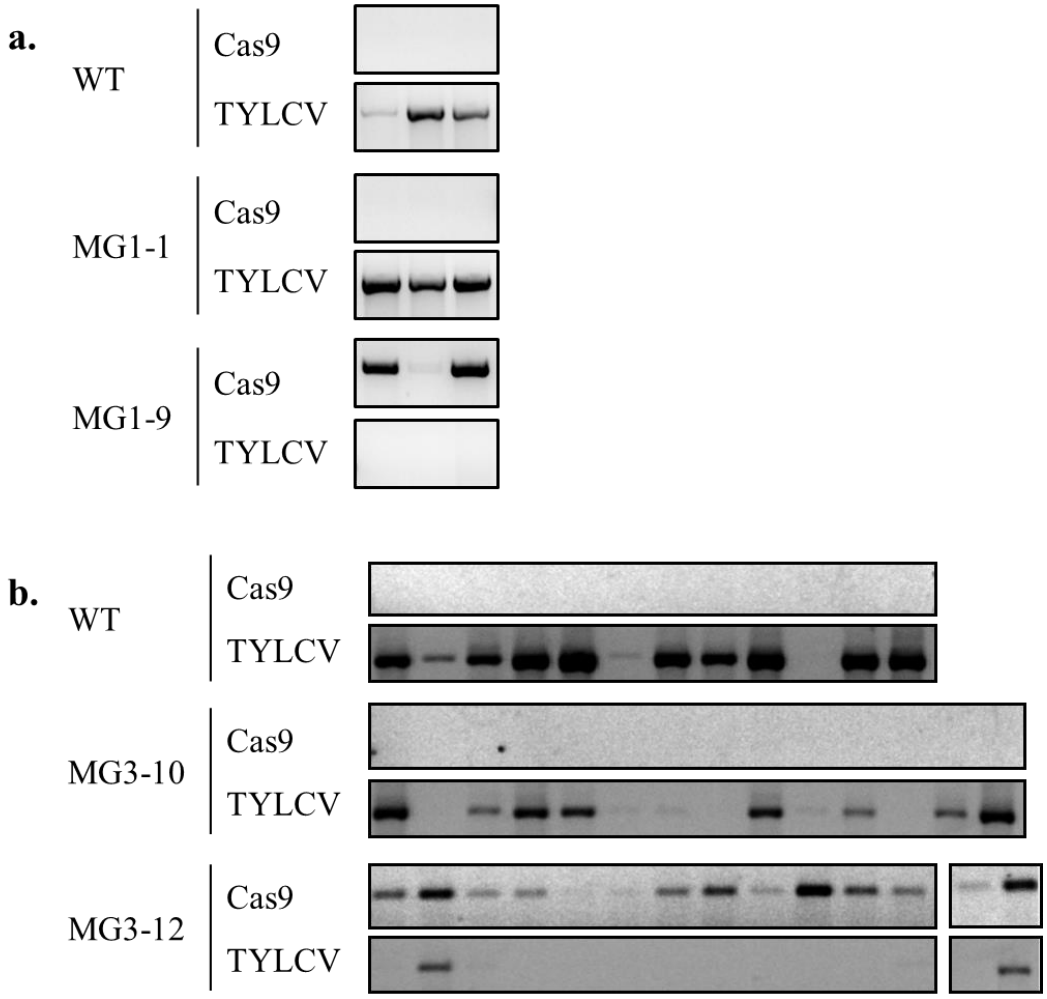

Figure 4-6. PCR screen analysis shows presence of Cas9 is generally associated with an absence of detectable TYLCV DNA. (a) MG1-1 and MG1-9 lines show an inverse relationship between the presence of Cas9 and TYLCV. (b) MG3 lines also show this relationship except for two plants that carry detectable levels of both Cas9 and TYLCV DNA. Wild-type, WT.

Discussion

Geminiviruses severely hamper crop health and food security in the face of a rapidly growing human population. The feasibility of CRISPR-mediated viral interference against this family of viruses has been demonstrated in the models *A. thaliana* and *N. benthamiana* (Ali *et al.*, 2015; Ali *et al.*, 2016). In this study, we extended the utility of this geminivirus control strategy to the major crop tomato. Through thoughtful CRISPR/Cas9 target selection, we designed sgRNAs that target a broad range of TYLCV isolates, avoid zones of high recombination frequency that may undermine durability of this strategy, and disrupt the DNA encoding the vital replication machinery. The functionality of these sgRNAs within our CRISPR/Cas9 plant expression vector was demonstrated through a transient *Agrobacterium*-

mediated *in planta* assay. Cas9-induced mutagenesis of the TYLCV genomic DNA fragment on a surrogate gemini-vector

The number of discovered *Geminiviridae* species continues to grow due to rapid evolution and advancing diagnostic techniques. As we discover and sequence more TYLCV isolates, the sgRNA arsenal may be changed and/or expanded to maximize CRISPR-mediated viral interference. Selection pressure is likely to change the target sequences. Such CRISPR evasion has already been observed in geminiviruses and phages (Ali *et al*., 2016; Paez-Espino *et al.*, 2015). Therefore, it is appropriate to expand the number of targets for a single TYLCV strain. More sites that are targeted by the CRISPR/Cas9 system translates into more genetic hurdles that the virus must overcome to escape Cas9 mutagenesis. In addition, Ali *et al*., 2016, found that targeting non-coding, intergenic sequences significantly limited the generation of CRISPR-evading viral variants capable of replication and systemic infection. Coupling of multiple sgRNA and specific targeting of non-coding, intergenic sequences could yield a more robust and durable form of CRISPR-mediated viral interference.

Interestingly, TYLCV DNA was observed in two samples that tested positive for the Cas9 transgene. Epigenetic changes, such as methylation, to the transgene sequence may induce silencing of Cas9, therefore halting CRISPR mediated viral interference (Vaucheret *et al.*, 1998). Western Blot analysis will elucidate whether silencing of the transgene occurred in this case. In addition, the inoculated TYLCV may have acquired two mutations that enabled it to evade matching the two sgRNAs.

At the time of TYLCV challenges, the transgenic lines of tomato were still segregating, likely resulting in some of the intermediate symptom score averages as some plants were lacking the Cas9 transgene. Future field trials of homozygous transgenic lines will demonstrate the durability of the assembled CRISPR/Cas9 expression systems. Cas9 evasion by change one Cas9 target site seems more feasible as opposed to simultaneously changing two Cas9 target sites. Therefore, the dual-targeting CRISPR transgenic lines are expected to exhibit more durable resistance to TYLCV infection and/or symptom development. How these transgenics will fare against mixed geminivirus challenges will depend on the sequences of the encountered TYLCV strains. As described above, continued discovery and sequence analysis of TYLCV isolates will bolster gRNA design for improved CRISPR-mediated viral interference. In addition, stacking of this viral immunity strategy with others such as aptamers, RNAi, and insect barriers can bolster resistance to Geminivirus infection.

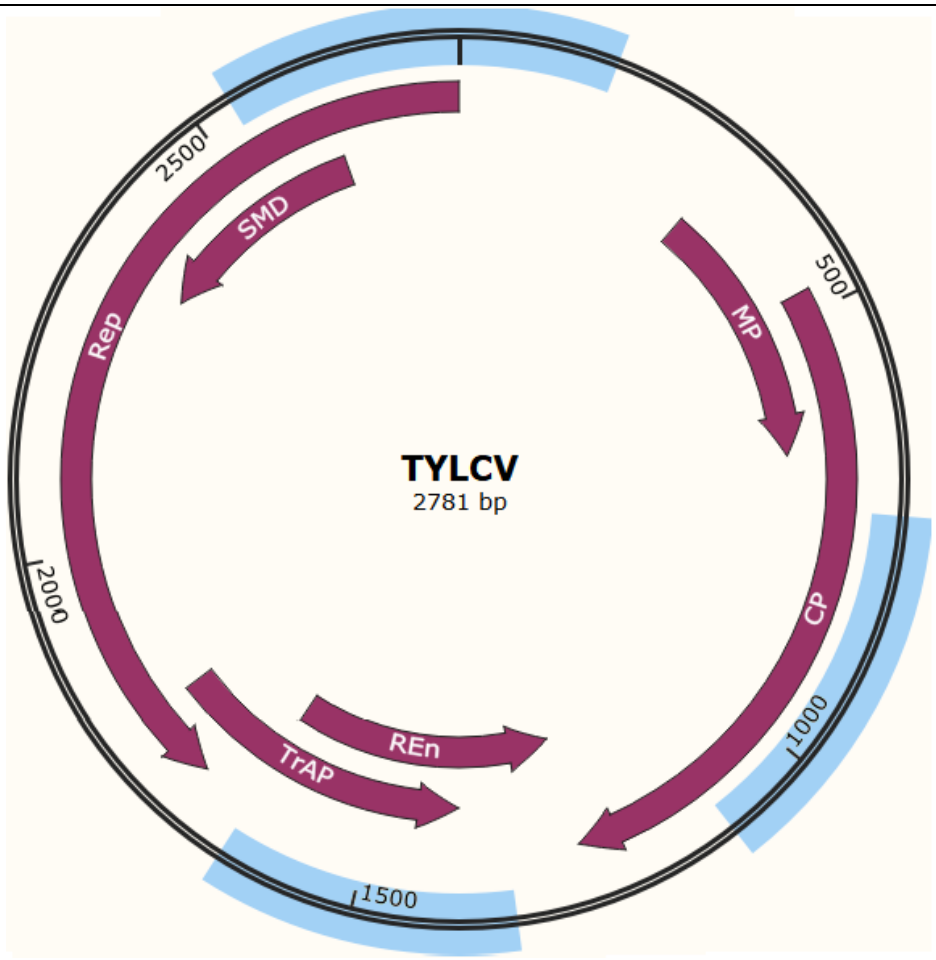

Figure S4-1. Map of TYLCV genome. Features annotated in purple. CP, coat protein; MP, movement protein; Rep, replication-associated protein; TrAP, transcriptional activator protein; REn, replication enhancer protein; SMD, symptom and movement determinant. Blue regions represent high recombination frequency zones.

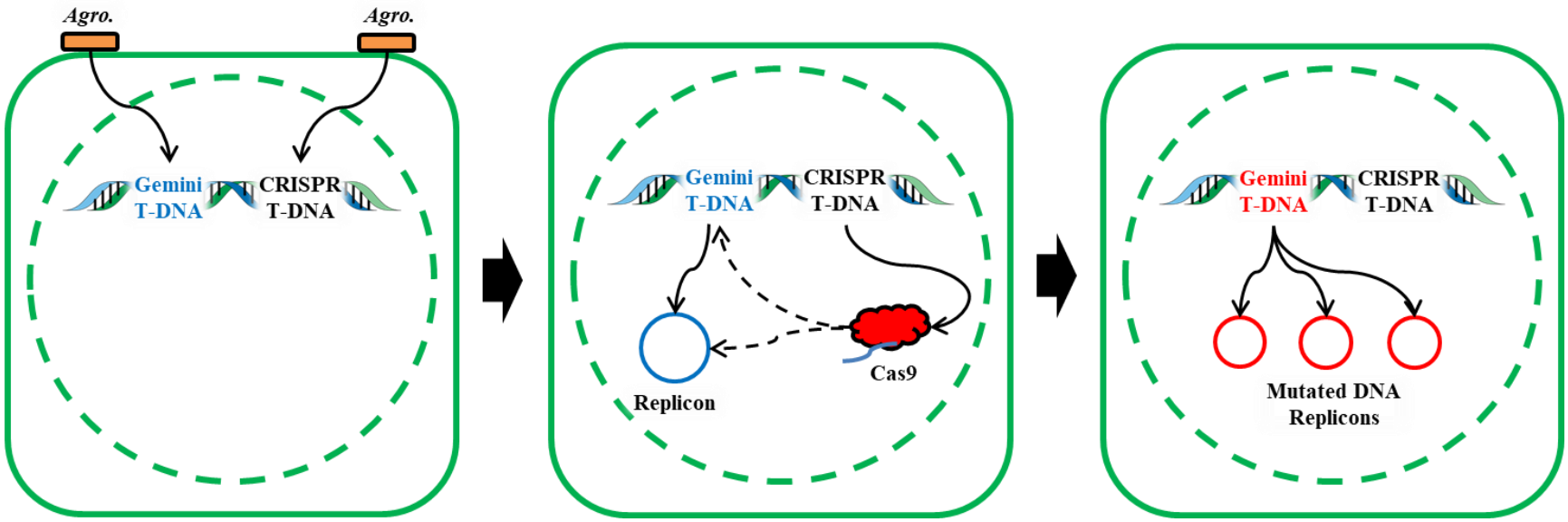

Figure S4-2. **Transient CRISPR/Cas9 *in planta* assay for targeting DNA sequences that are not endogenous to *N. benthamiana***. Agrobacterium strains carrying the CRISPR/Cas9 expression vector and gemini-vector bearing the foreign DNA of interest co-deliver their respective T-DNAs into the plant genome. Following integration, gemini T-DNA releases and replicates DNA replicons carrying the Cas9 target. CRISPR T-DNA expression yields complete Cas9/sgRNA complexes that are directed toward the matching DNA of interest. Cas9 target the replicons or the gemini T-DNA itself. Following Cas9-induced mutagenesis of the gemini T-DNA, DNA replicons bearing the mutation continue to be released and replicated.

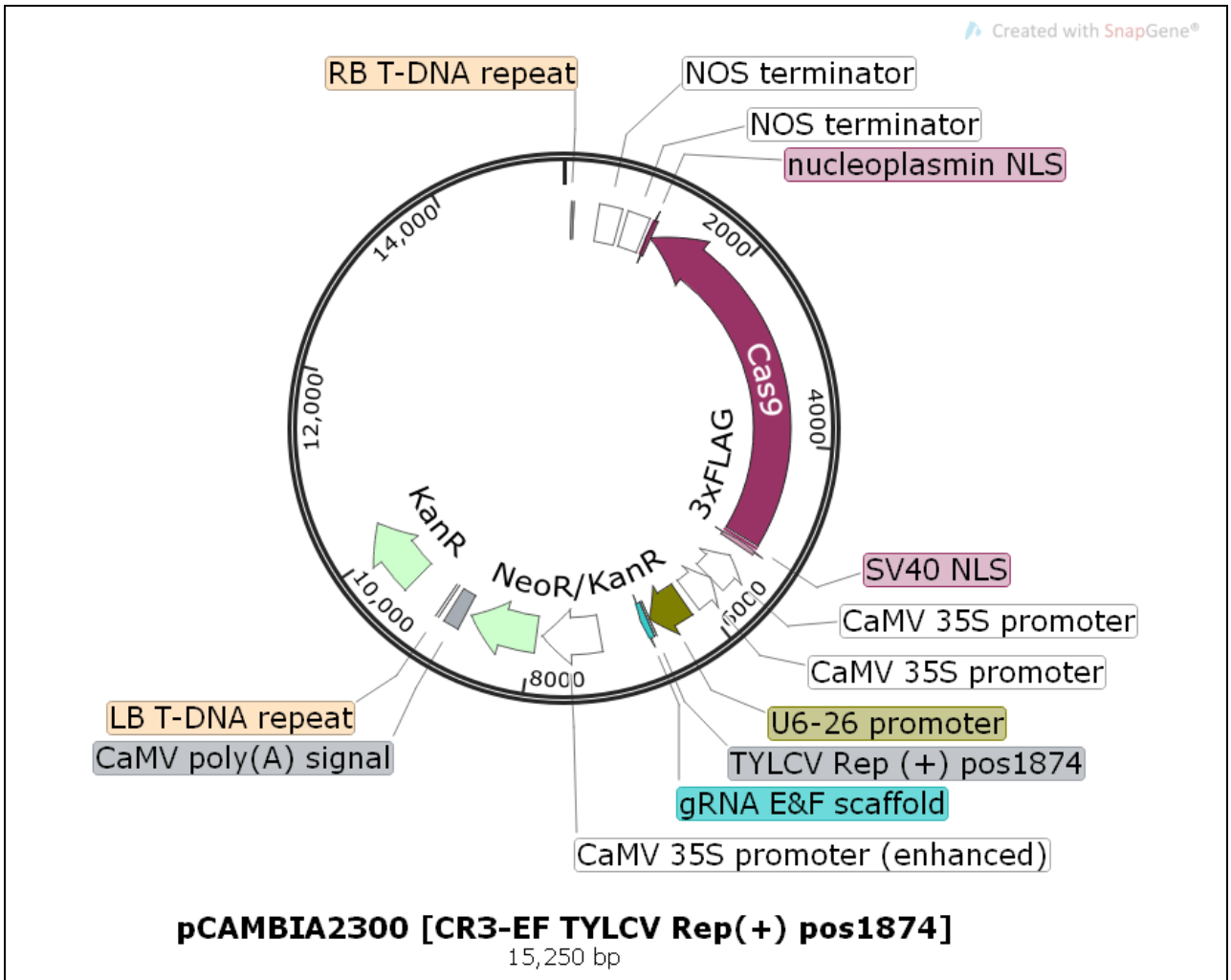

Figure S4-3. Map of *CRISPR/Cas9* plant expression vector targeting TYLCV Rep(+) pos.1874.

Figure S4-4. Sequence of *CRISPR/Cas9* plant expression T-DNA targeting TYLCV Rep(+) pos.1874.

GTTTACCCGCCAATATATCCTGTCAAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCTGATCCAAGCTCAAGCTGCTC
TAGCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGG
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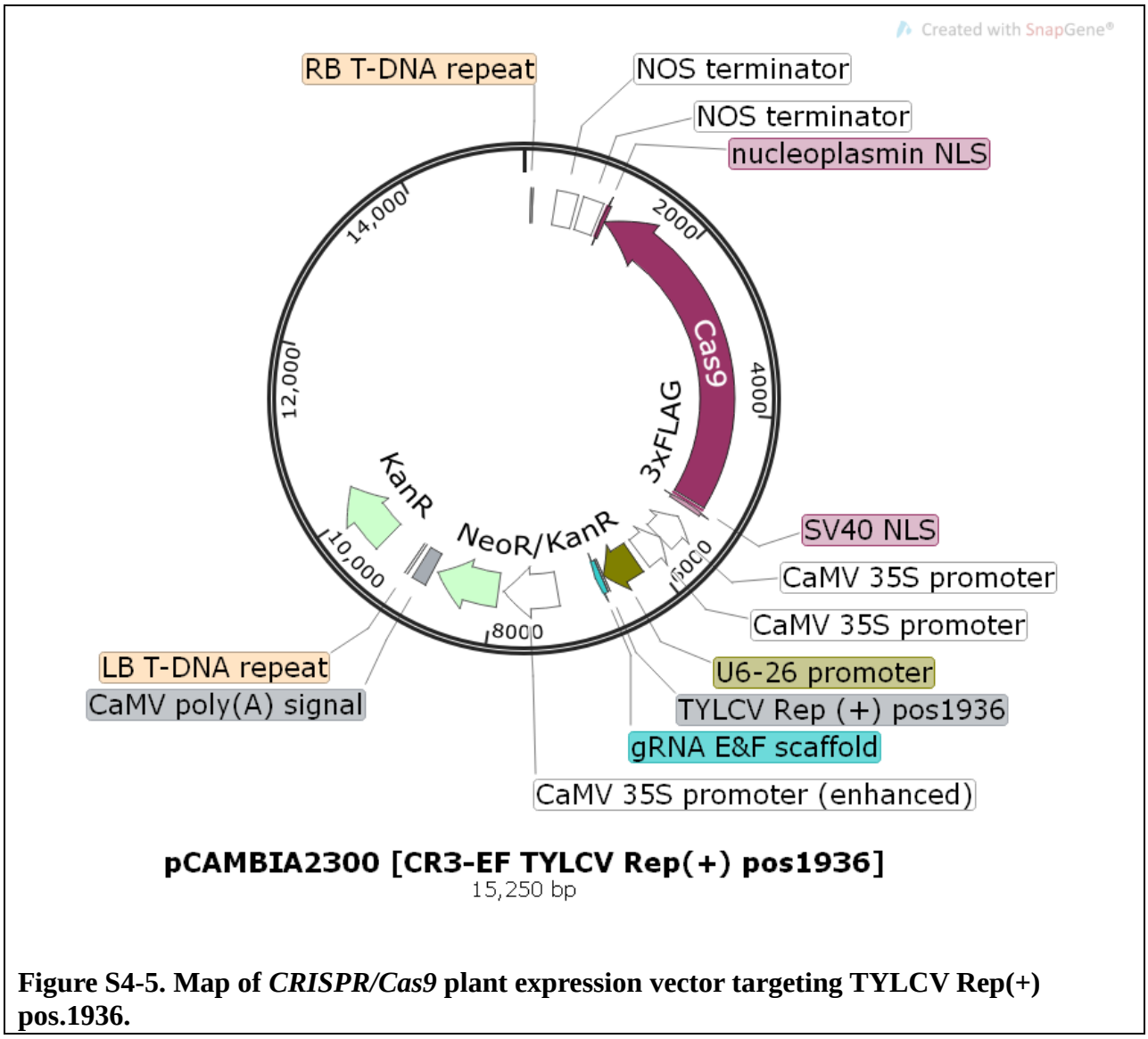

Figure S4-5. Map of *CRISPR/Cas9* plant expression vector targeting TYLCV Rep(+) pos.1936.

Figure S4-6. Sequence of *CRISPR/Cas9* plant expression T-DNA targeting TYLCV Rep(+) pos.1936.

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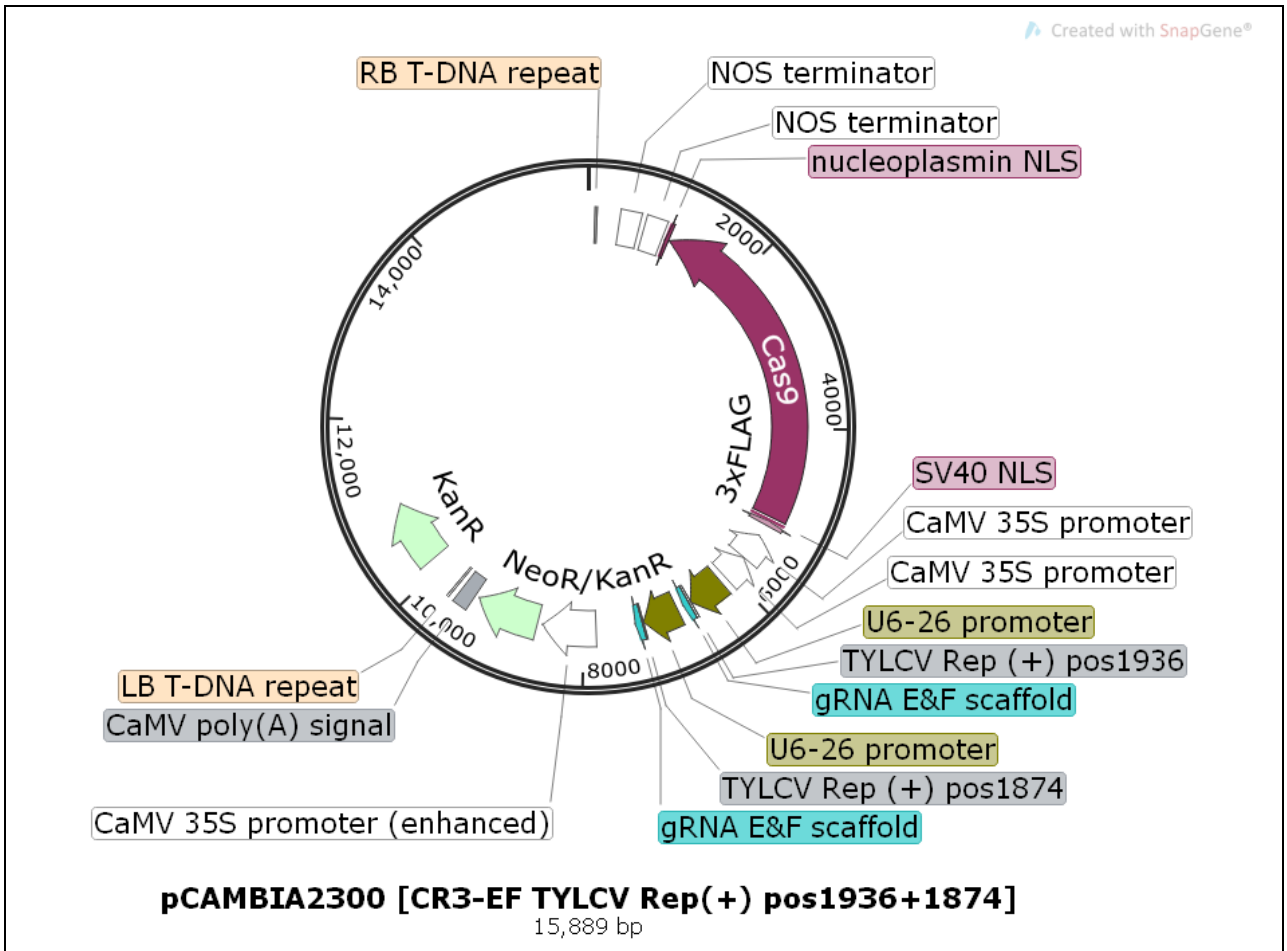

Figure S4-7. Map of *CRISPR/Cas9* plant expression vector targeting TYLCV Rep(+) pos.1936 and pos.1874.

Figure S4-8. Sequence of *CRISPR/Cas9* plant expression T-DNA targeting TYLCV Rep(+) pos.1936 and pos.1874.

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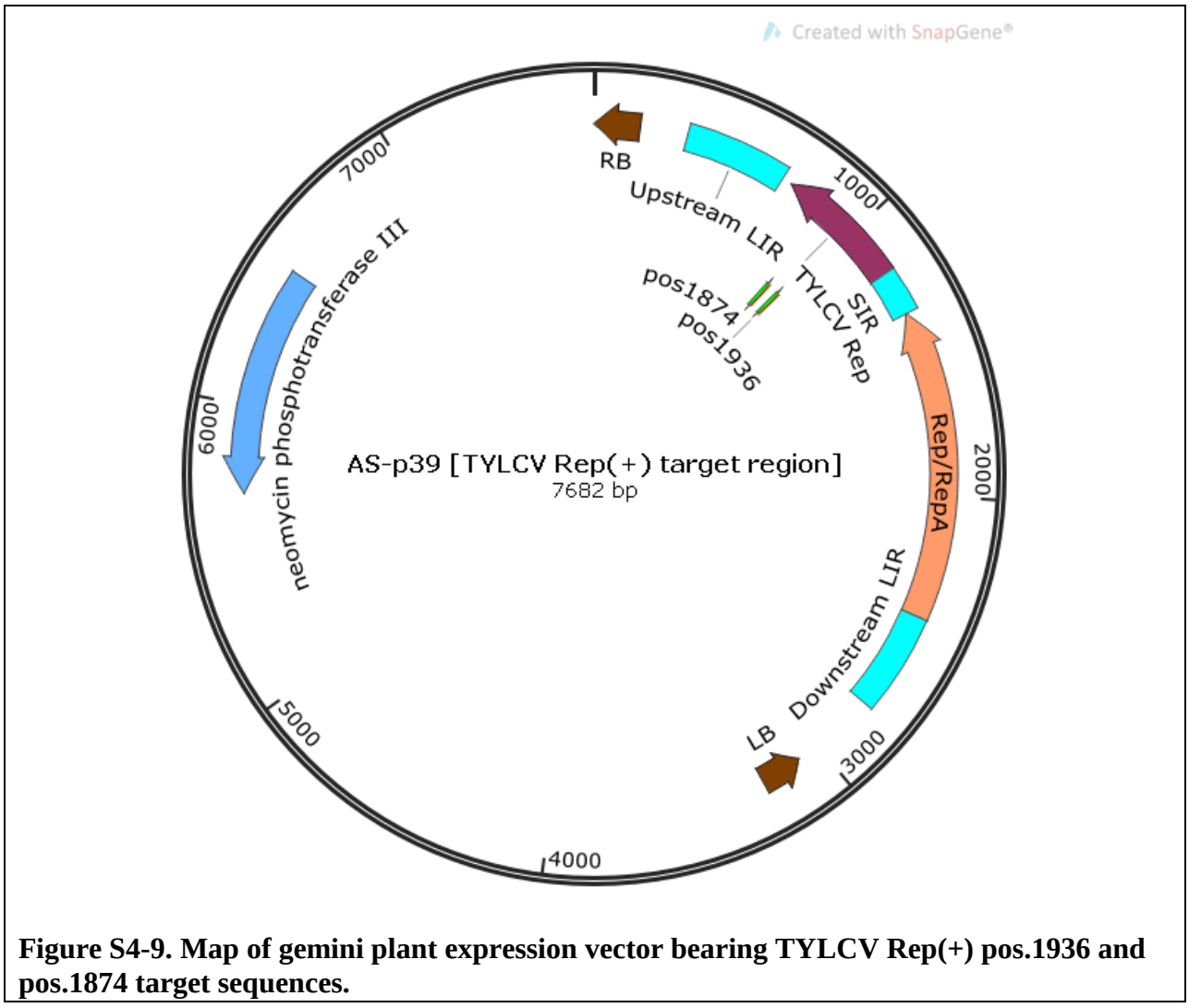

Figure S4-9. Map of gemini plant expression vector bearing TYLCV Rep(+) pos.1936 and pos.1874 target sequences.

Figure S4-10. Sequence of gemini plant expression vector bearing TYLCV Rep(+) pos.1936 and pos.1874 target sequences. TYLCV Rep target region in capital letters.

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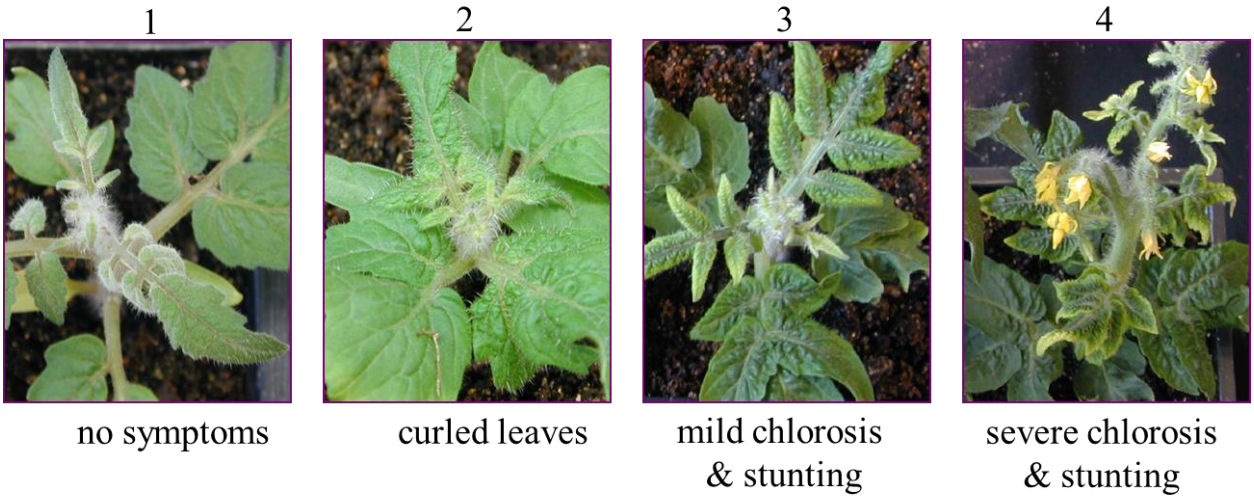

Figure S4-11. TYLCV symptom scoring scheme on a scale of 1-4.

5. Future directions

CRISPR-mediated viral susceptibility gene mutagenesis and viral interference reflect the vast potential of the CRISPR technology as a solution to global food challenges. Viruses of the *Potyviridae* and *Geminiviridae* families pose serious threats to crop yield and food security worldwide. Here, we demonstrated that targeting of VPg-interacting eIF4E isoforms conferred suppression of CBSD and TuMV infection in cassava and *A. thaliana*, respectively. In addition, CRISPR-mediated viral interference of TYLCV in tomato significantly impaired symptom development and viral accumulation. A developing understanding of host-pathogen interactions and technological advances will continue to shape and refine these disease resistance strategies.

Future work on genetically engineering *Potyviridae* resistance would benefit by shifting from the CRISPR-mediated knockout strategy toward developing functional resistance alleles. eIF4E and its isoforms exhibit varying degrees of functional redundancy and specialization. For example, *A. thaliana* eIF(iso)4E is specifically involved in the response to phosphate deprivation through selective translation of a phosphate transporter (Martinez-Silva *et al.*, 2012). In contrast, little is known regarding the specific function of nCBPs. Knocking out the nCBPs in cassava may carry some impact on fitness that was not observed under greenhouse and laboratory conditions. Similar to *A. thaliana* eIF(iso)4E, the cassava nCBPs may have specific roles in response to certain stimuli, in which case it would be optimal to maintain their function. As discussed in Bastet *et al*., 2017, it is also impractical to pyramid genetic resistances to multiple *Potyviridae* through knocking out multiple eIF4E isoforms. In the case of *A. thaliana*, different *Potyviridae* species selectively hijack eIF4E1 or eIF(iso)4E for survival (Sato et al., 2005). An *eif4e1*/*eif(iso)4e* double mutant in *A. thaliana* resulted in male gametophyte lethality, preventing transmission of resistance to progeny (Callot et al., 2014). In the event our *ncbp-1*/*ncbp-2* mutant cassava is faced with a resistance-breaking CBSV variant or another *Potyviridae* species that hijacks an alternative eIF4E isoform, additional eIF4E isoform knockouts may likewise have deleterious effects on cassava fitness. Rather than knocking out eIF4E isoforms, we can pursue employment of functional resistance alleles. There are multiple cases of natural potyvirus resistance arising from point mutations that do not impair translation initiation, including *Lycopersicum* spp. (*pot1*), *Capsicum* spp. (*pvr2*), and *Cucumis melo* (*nsv*) (Bastet *et al*., 2017). Resistance alleles can be engineered in other crops via precise gene editing using a donor template that bears the desired mutation. Upon cleavage of the target allele, the donor template may be integrated via homology directed repair. Naturally-occurring functional resistance alleles have not been found in cassava, likely due to clonal propagation and breeding challenges (Ceballos *et al.*, 2004). Further analysis of nCBP structure and function is needed to determine which regions may be modified for disease resistance without impacting its activity.

CRISPR-mediated editing of susceptibility genes is also heading in the direction of DNA-free methods of delivery. Gene editing using preassembled Cas9-gRNA ribonucleoproteins (RNPs) has been accomplished in tobacco, maize, and other major crops (Woo *et al*., 2015; Svitashev *et al.*, 2016). RNP delivery methods include polyethylene glycol (PEG)-mediated transfection and biolistic delivery on gold particles. Upon entry into the nucleus, RNPs may dissociate from gold particles and carry out gene targeting. Traditional transgenic methods risk disruptive integration of the foreign DNA into the plant genome, complex plant mosaicism arising from CRISPR activity after cell division, and off-target effects (Jia *et al*., 2012; Svitashev

et al., 2016; Woo *et al*., 2015). Off target mutations may be segregated away in subsequent generations, but this approach is not practical with crops such as cassava due to long reproduction cycles and high heterogeneity. DNA-free approaches attractively minimize the potential for these risks. RNPs cleave target sites immediately after transfection and are degraded by cellular proteases (Woo *et al*., 2015). The transient nature of this DNA-free method leaves no footprint besides the intended gene edit. Project design workload is also minimized as DNA-free approaches eliminate the need to codon optimize Cas9 for expression in a particular organism. Furthermore, DNA-free methods are advantageous under the current government regulatory framework. The United States Department of Agriculture recently exempted CRISPR/Cas9-edited mushroom and corn from restrictive genetically modified organism (GMO) regulation (Globus and Qimron, 2017). These crops are non-transgenic, and were engineered without the plant pest *Agrobacterium tumefaciens*. DNA-free methods would likewise fall under this exemption. The current regulatory landscape and precision of transient RNPs favor the growth of DNA-free approaches to modifying crops.

The efficacy and extended utility of CRISPR-mediated viral interference will be supported by improved molecular techniques for the discovery of new *Geminiviridae* species. Innovative approaches such as the vector-enabled metagenomics (VEM) technique, which uses insect vectors as viral sampling tools, offer additional means to characterize the diversity of *Geminiviridae* species afflicting crop production (Rosario *et al*., 2013). Coupled with the falling cost of sequencing, these techniques will rapidly reveal optimal CRISPR/Cas9 targets for broad-spectrum viral interference. These newly discovered targets can be stacked with other gRNA on a plant CRISPR expression system. This strategy applies great selective pressure for viral species to evade CRISPR/Cas9 recognition by modifying the targeted DNA sequence. The potential for resistance-breaking variants mandates close monitoring of virus species in the field. However, the ability of *Geminiviridae* species to generate CRISPR/Cas9-evasive variants seems limited. Ali *et al*., 2016, found that targeting of the non-coding intergenic regions resulted in viral interference and inefficient virus variant generation. Future CRISPR-mediated viral interference efforts may focus on targeting these intergenic regions. The versatile multiplexing ability of the CRISPR/Cas9 coupled with targeted focus on these intergenic regions can offer durable and robust disease resistance.

The CRISPR system has revolutionized plant genome engineering for disease resistance. As technological advances are made in the use and delivery of this system, the speed and cost of generating disease-resistant crop varieties will continue to drop. With a growing understanding of proteins involved in plant-pathogen interactions, researchers will be able to engineer precise edits that impair infection without any potential sacrifice to crop fitness and yield. Field testing of these CRISPR-modified crops will elucidate the effectiveness of these strategies and where they may be improved. Sustainable food production for our global population will continue to be bolstered by the innovative and precise application of the CRISPR technology.

6. Materials and Methods

Chemically competent *Agrobacterium* preparation

Agrobacterium tumefaciens strain GV3101 were grown overnight in 2ml culture with 50 mg/ml rifampicin and 5 mg/ml gentamycin at 28°C, 250 rounds per minute. Overnight culture was transferred into 50 ml culture with selection and grown to OD_{600}=0.8. Cells were pelleted for 5 minutes at 5,000 x g. Media was decanted, and the pellet was resuspended in 1 ml of ice cold 20 mM $CaCl_2$. 50-µl aliquots were stored at −80°C.

A. thaliana growth conditions and transformation

A. thaliana accession Columbia-0 was used as wild-type control and for transformation. Individual plants were grown in pots with SuperSoil mix and were kept at 21°C with an 8-h light and 16-h dark photoperiod for 6 weeks before infection.

Chemically competent *A. tumefaciens* cells were transformed with appropriate CRISPR constructs by snap freezing, and transformed cultures were grown overnight in Luria-Bertani (LB) liquid medium at 28°C. Cells were pelleted by centrifugation at 5000 x g for 15 minutes. Pelleted cells were washed and resuspended in liquid LB to OD_{600} of 0.8 in 5.0% sucrose, 0.05% Silwet L-77. Floral dips were performed as described in Clough and Bent, 1998. Flowering *A. thaliana* were dipped in suspension and gently agitated by tapping. Plants were covered overnight to retain humidity after inoculation. Floral dips were repeated six days later.

Transgenic *A. thaliana* kanamycin selection

Seeds were sterilized by a modified vapor-phase method (Clough and Bent, 1998). Briefly, an open container of seeds was placed inside a desiccator jar in a fume hood. Within the desiccator jar, 3 ml HCl was added to 30 ml 100% bleach. The jar was then sealed for 3 hours for seed surface sterilization. Sterilized seeds were spread evenly on solid agar plates with kanamycin selection (4.3 g/L Murashige & Skoog Salts, 20 g/L Sucrose, 0.5 g/L MES hydrate, 8 g Bacto Agar, pH adjusted to 5.7 with KOH; autoclave for 30 minutes).

TuMV-GFP viral propagation and infection

TuMV-GFP infectious clone was gifted to us by the laboratory of James Carrington at the Donald Danforth Plant Science Center. TuMV-GFP propagation and infections were conducted as described in Casteel *et al.*, 2014.

Production of cassava plants and growth conditions

Transgenic cassava lines of cultivar 60444 were generated and maintained *in vitro* as described previously (Taylor *et al.*, 2012). *In vitro* plantlets were propagated, established in soil, and transferred to the greenhouse (Taylor *et al.*, 2012; Wagaba *et al.*, 2013). Throughout the course of a disease trial, all plants were treated bi-weekly for pest control by gently spraying the undersides of all leaves with water.

Identification and phylogenetic analysis of eIF4E isoforms

BLAST search of the AM560-2 cassava cultivar genome was done via Phytozome V10 using *A. thaliana* eIF4E family proteins as the queries (Goodstein *et al*., 2012). The coding sequences of each isoform were verified by comparison to RNA-seq data (Cohn *et al.*, 2014). Clustal Omega (EMBL-EBI) was used to generate the percent identity matrix of all eIF4E isoform amino acid sequences (Goujon *et al.*, 2010; Sievers *et al.*, 2014). MEGA 6 software was used to generate a phylogenetic tree of the cassava and *A. thaliana* eIF4E isoforms (Tamura *et al*., 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel, 1993). This amino acid substitution model was determined as best fit using the MEGA 6 model test. The tree with the highest log likelihood (-2025.7966) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.9218)). The analysis involved 9 amino acid sequences. All positions containing gaps and missing data were eliminated.

CRISPR/Cas9 binary construct design

CRISPR/Cas9 construct design and assembly of entry clone pCR3 were conducted as described by Paula de Toledo Thomazella *et al.* (2016). CRISPR/Cas9 constructs targeting two sites were assembled via Gibson Assembly of the other U6-26/sgRNA into the *Sac*II site of the entry clone. Flanked by the attL1 and attL2 recombination sequences, the cassette carrying the Cas9/sgRNA expression system was Gateway cloned into the binary destination vector pCAMBIA2300 (Hajdukiewicz *et al.*, 1994).

sgRNA design and cloning

Target sequences were identified in *nCBP-1* and *nCBP-2* genes of cassava using the online CRISPR-P software (Lei *et al.*, 2014). This tool was used to select targets with predicted cut sites within exons, minimal off-target potential, and overlapping restriction enzyme recognition sites.

sgRNA forward and reverse primers were designed with overhangs compatible with the *Bsa*I-site described above. The Golden Gate (GG) cloning method was used to *Bsa*I digest the pCR3 vector and ligate in the sgRNA. In the case of the dual targeting CRISPR/Cas9 construct, the pCR3 vector bearing sgRNA1 was digested with SacII, a site within the LR clonase *attL* sequences. The *A. thaliana* U6-26 promoter and sgRNA2 were PCR-amplified using primers suitable for Gibson Assembly into the *Sac*II cut site of the digested pCR3-sgRNA1 vector. For Gibson Assembly, 100 ng of *Sac*II-digested vector was incubated with 200 ng of U6-26p-sgRNA2 PCR amplicon and Gibson Assembly Master Mix for one hour and transformed into *E. coli* (NEB5a). Sequences of cloned CRISPR constructs were verified via Sanger sequencing.

Yeast two-hybrid

The *eIF4E* isoforms were amplified by PCR using primers suitable for Gibson Assembly into the *Bam*HI site of pEG202. Yeast codon optimized coding sequences of the CBSV and UCBSV VPg were synthesized through Genewiz, Inc. (South Plainfield, NJ, USA). The VPg coding sequences were amplified using primers suitable for Gibson Assembly into the *Eco*RI site of pEG201. Yeast two-hybrid analyses were carried out as described previously (Kim *et al.*, 2014).

Genotyping and mutant verification

100 mg of leaf tissue was collected from plant tissue and genomic DNA extracted using the CTAB extraction procedure (Murray and Thompson, 1980). Transgenic plants were genotyped for Cas9-induced mutagenesis via RESL and Sanger sequencing (Voytas, 2013). Initially, 100 ng of genomic DNA was PCR amplified using primers encompassing the Cas9 target sites. PCR amplicons were gel purified on 1.5% agarose gel and purified with the QIAquick Gel Extraction Kit. For RESL analysis, 50 ng of PCR amplicon were digested with restriction enzyme *Sml*I for 12 hours, then run and visualized on a 1.5% agarose gel. For genomic and cDNA sequence analysis, the amplicons were subcloned and Sanger sequenced through the UC Berkeley DNA Sequencing Facility. Between six to eight clones were sequenced to discriminate INDEL polymorphisms and sequences were aligned to the intact *nCBP-1* and *nCBP-2* using SnapGene software (from GSL Biotech; available at snapgene.com).

CBSV and UCBSV inoculation and disease scoring

Prior to virus challenge, micropropagated cassava plantlets were transplanted to soil, allowed to acclimate for six to eight weeks, and chip-bud graft inoculation performed as described previously (Wagaba *et al.*, 2013). Briefly, one plant of each genotype received an axillary bud from a single previously infected wild type plant, resulting in one inoculation cohort. Multiple cohorts were used in a single experiment to control for donor plants with varying viral concentrations.

Shoot tissues were scored two to three times a week for 12 to 14 weeks. Leaves and stems were each scored on separate 0-4 scales (Table 3-2). Leaf and stem scores were then summed to generate an overall aerial severity score for a particular time point. These data were used to calculate the area under the disease progression curve (Simko and Piepho, 2012). To assess symptom severity in storage roots, each storage root was evenly divided into five pieces along its length. Each storage root piece was then sectioned into one-centimeter slices and the maximum observed severity was used to assign a symptom severity score to that storage root piece. The scores for all storage root pieces of a given plant were then averaged to determine the overall severity score.

Storage root viral titer quantification

Five to ten representative storage root slices per plant were collected for viral titer quantification. Samples were flash frozen in liquid nitrogen and lyophilized for two days. Lyophilized storage roots were pulverized in 50 mL conical tubes with a FastPrepTM-24

instrument (MP Biomedicals) and 75 mg of pulverized tissue was aliquoted into Safe-Lock microcentrifuge tubes (Eppendorf) pre-loaded with two mm zirconia beads (BioSpec Products). Samples were flash frozen in liquid nitrogen, further homogenized to a finer consistency, and one mL of Fruit-mate (Takara) added to each sample. Samples were homogenized and subsequently centrifuged to remove debris. The supernatant was removed, mixed with an equal volume of TRIzol LS (Thermo Fisher), and the resulting mixture processed with the Direct-zol RNA MiniPrep kit (Zymo Research). Resulting total RNA was normalized to a standard concentration and used for cDNA synthesis with SuperScript III reverse transcriptase (Thermo Fisher).

Quantitative PCR was done with SYBR Select Master Mix (Thermo Fisher) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Primers specific for CBSV-Nal *HAM1-LIKE* and cassava *PP2A4* were used for relative quantitation. Normalized relative quantities were calculated using formulas described by Hellemans *et al.* (2007). For combined analysis of all experimental replicates, normalized relative quantities for all samples were further normalized as a ratio to the geomean of wild type for their respective experiments. Data were then pooled and a Mann-Whitney U test was used to assess statistical differences.

Tomato plant growth and transformation

Agrobacterium-mediated transformation and regeneration of tomato were conducted as described by de Toledo Thomazella *et al.*, 2016.

Transient assays in *N. benthamiana* for evaluation of gRNA efficiency

gRNA efficiency was evaluated as described by de Toledo Thomazella *et al*., 2016.

***Agrobacterium*-mediated inoculation of tomato with TYLCV**

Tomato plants were infected with TYLCV as described by Reyes *et al*., 2013.

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