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Electronic Supplementary Material

Conferring Resistance to Plant RNA Viruses with the CRISPR/CasRx System

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Supplementary materials

Materials and Methods

Plant materials and growth conditions

Wild-type *Nicotiana benthamiana* was potted in soil and placed in an insect-free growth chamber at 25°C and 60% relative humidity greenhouse under a 16 h light/8 h dark photo-period.

Plasmid construction

Construction of pCambia1300-CasRx/dCasRx recombinant plasmids

We designed a recombinant construct pCambia1300-CasRx (CasRx), which was based on the pCambia1300 backbone vector, including the enhanced Pol III promoter 35S (e35S) from the cauliflower mosaic virus (CaMV), 3×Flag tag in the N-terminal, a plant-codon-optimized *CasRx* gene fused with 2×nuclear localization signal (NLS) sequences (Sequence 1), and the Nos terminator (Fig. 1A). The information of nucleotide sequences and amino acid sequences of plant-codon-optimized *CasRx* is based on the previous report (Konermann et al., 2018). We artificially synthesized the dsDNA fragments of Sequence 1 by gene synthesis services provided by Beijing Qingke Biotechnology Co., Ltd. The DNA fragments were cloned into the pUC19 plasmid, and then were subcloned into pCambia1300-3*Flag vector by *Pst* I and *Bam*H I. The pCambia1300-3*Flag -dCasRx (dCasRx) plasmid was constructed using the similar strategy, and a mutant version of the CasRx protein by replacing the key specific residue motif R-X_4-H with A-X_4-A by using the KOD-plus-mutagenesis kit (Toyobo) with the specific

mutagenesis primers (listed in Sequence 2).

Construction of crRNA expression constructs

To construct the crRNA expression clones, the ssDNA oligonucleotides of CasRx corresponding direct repeat with the targeting or non-targeting (GUS) sequences were synthesized from Beijing Qingke Biotechnology Co.,Ltd as (all crRNA sequences used in this study are listed in Fig. 1C). The phosphorylated and annealed dsDNA fragments of crRNAs were ligated into the pAtU6:Vec based on pUC57 backbone by a *Bsa* I restriction site under the Arabidopsis snoRNA U6 promoter, or were inserted into tobacco rattle virus (TRV)-based vector under the PEBV promoter using *Xba* I and either *Xho* I or *Bam*H I restriction sites.

Viral strains

Agro-infectious clones of TuMV-GFP (Cotton et al., 2009), TMV-GFP (Shivprasad et al., 1999), and CMV-GFP (Fny strain) (Liao et al., 2015) were stored in our lab, and were described previously.

Agro-infiltration of *N. benthamiana* leaves and GFP imaging

For transient expression analysis in *N. benthamiana* leaves, constructs were generated in binary vectors and transformed into *Agrobacterium tumefaciens* strain EHA105 via electroporation. Overnight-grown single colonies in selective medium were centrifuged and suspended in infiltration buffer (10 mmol/L MES, pH 5.7, 10 mmol/L $CaCl_2$, and 100 μmol/L acetosyringone dissolved in DMSO), and then were mixed at the same ration. After a 2 h incubation at room temperature, the mixed *Agrobacterium* cultures carrying different constructs were infiltrated into leaves of *N. benthamiana* plants at the 4–5 leaf stage with a 1mL needleless syringe. The infiltrated plants were photographed with a Canon 50D digital camera using an ultraviolet handheld light at different time periods at a high ISO speed capture. The negative control plants were agroinfiltrated with infiltration buffer as mock inoculations. The infiltration assay experiments were repeated at least three times, and the infiltration leaves or systemically infected leaves were collected at different time points for further experiments.

Immunoblot analysis

Total protein was extracted from infiltrated leaf patches or systemically infected leaves using urea protein extraction buffer (50 mmol/L Tris-HCl, pH 6.8, 9 mol/L urea, 4.5% SDS and 7.5% β-mercaptoethanol, and 1 mmol/L EDTA). Proteins were separated on a 10% SDS-PAGE electrophoresis. Immunoblot analysis was conducted using primary mouse monoclonal antibodies (the GFP monoclonal antibody at a dilution of 1:8000 was obtained from Roche, the Flag monoclonal antibody at a dilution of 1:5000 was obtained from Sigma, and the TuMV CP monoclonal antibody at a dilution of 1:10000 was produced in our lab). For note, the secondary antibody was a goat anti-mouse IgG conjugated with peroxidase at 1:10000 dilution (Cell Signaling Technology). Blotted membranes were washed thoroughly and were detected by chemiluminescence using a high-signal ECL western blotting substrate (Tanon) by a chemiluminescence detection system (Tanon).

RT-qPCR Analysis

Total RNA was extracted from systemic leaves infected by TuMV-GFP, CasRx together with mock (infiltration buffer), or TRV-crGUS, or TRV-crGFP at 7 dpi using Trizol reagent (Invitrogen), and 1 μg of RNA was used for cDNA synthesis by using Oligo(dT)12-18 primer or specific primers using the PrimeScript^{TM} reagent kit with gDNA eraser (TaKaRa) according to the manufacturer's protocol. The RT-qPCR was performed in triplicates using a Roche Light Cycler 96 system (Roche). The specificity of primer pairs was verified by RT-qPCR dissociation curve. The relative expression level was calculated using the comparative Cq ($2^{-\Delta\Delta Cq}$) method. *NbActin* was used as an internal standard. These data were analyzed using two-sided Student's t-test, $^{**}P < 0.01$.

References:

Cotton S, Grangeon R, Thivierge K, Mathieu I, Ide C, Wei T, Wang A, Laliberté JF (2009) Turnip mosaic virus RNA replication complex vesicles are mobile, align with microfilaments, and are each derived from a single viral genome. J Virol 83:10460-10471.

Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD (2018) Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. Cell 173:665-676.

Liao Q, Tu Y, Carr JP, Du Z (2015) An improved cucumber mosaic virus - based vector for efficient decoying of plant microRNAs. Sci Rep 5:13178.

Shivprasad S, Pogue GP, Lewandowski DJ, Hidalgo J, Donson J, Grill LK, Dawson WO (1999) Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus–based vectors. Virology 255:312-323.

Sequence 1: NLS-CasRx-NLS:

Nucleotide sequences:

ATGTCTCCGAAAAAAAAAAGGAAGGTCGAGGCGAGCATTGAGAAAAAGAAGAGCTTTGCTAAAGGT
ATGGGGGTTAAGAGTACATTGGTGTCCGGTTCCAAAGTGTATATGACTACATTTGCGGAAGGCAGCGA
CGCAAGGCTGGAGAAAATAGTTGAAGGCGATTCAATAAGATCGGTGAATGAAGGAGAAGCCTTCTCC
GCTGAAATGGCCGACAAAAATGCCGGTTATAAGATTGGTAACGCTAAGTTCTCGCACCCAAAGGGTTA
TGCAGTCGTGGCGAACAATCCACTGTACACAGGTCCTGTGCAGCAGGATATGCTTGGTTTGAAGGAA
ACGTTGGAAAAGCGGTACTTCGGTGAGTCCGCTGACGGCAATGATAATATATGTATTCAGGTGATACA
CAACATCCTCGATATAGAAAAAATCCTCGCTGAGTACATTACAAATGCCGCCTACGCGGTCAATAACAT
ATCCGGTTTGGACAAAGACATCATAGGATTTGGAAAATTCTCTACAGTGTACACGTACGATGAATTTAA
AGACCCGGAGCATCACAGAGCAGCTTTCAACAACAATGATAAGCTCATAAATGCAATAAAAGCGCAA
TATGATGAATTTGACAACTTTCTTGATAACCCGAGGCTGGGCTATTTTGGTCAGGCTTTCTTCAGCAAA
GAAGGGCGCAACTATATTATAAATTACGGCAATGAGTGCTATGATATTTTGGCATTGTTGTCCGGACTG
CGGCATTGGGTTGTTCATAATAACGAGGAAGAGAGTAGGATCAGTCGCACATGGCTGTATAACCTTG
ATAAGAATCTCGATAATGAGTATATTTCGACTTTGAACTACTTGTATGATCGCATTACTAACGAACTCAC
CAACAGCTTTTCGAAGAACTCGGCAGCCAACGTGAATTATATAGCGGAAACCTTGGGGATTAACCCA
GCAGAATTCGCAGAACAGTACTTTCGCTTCAGCATAATGAAGGAACAAAAGAACCTCGGTTTTAACAT
CACAAAACTCAGAGAGGTTATGCTGGATAGAAAAGACATGTCAGAAATTCGGAAAAATCATAAAGTG

TTCGATTCCATCAGGACGAAGGTCTACACAATGATGGATTTCGTCATCTACAGATATTATATTGAGGAG
GATGCAAAAGTCGCAGCGGCCAACAAAAGCCTCCCTGACAATGAAAAGTCGCTCTCTGAAAAGGAC
ATCTTTGTCATAAACCTTCGGGGCAGTTTTAACGATGACCAAAAGGACGCCTTGTACTACGATGAAGC
AAATCGCATCTGGAGGAAACTTGAGAACATAATGCATAACATAAAGGAGTTTCGGGGGAACAAGACG
AGAGAATACAAAAAGAAGGACGCGCCAAGACTTCCTAGAATTCTCCCAGCGGGGCGCGACGTCTCA
GCGTTCTCCAAGCTCATGTACGCGCTTACCATGTTCCTCGATGGAAAAGAGATAAATGATCTTTTGACT
ACGCTCATTAACAAGTTCGACAACATTCAATCTTTCCTGAAAGTGATGCCTCTCATAGGGGTCAACGC
AAAGTTCGTTGAAGAATACGCCTTTTTCAAGGACTCTGCGAAGATAGCCGATGAACTCCGCCTCATAA
AGAGCTTTGCGCGGATGGGTGAACCTATTGCTGACGCCCGGAGGGCAATGTATATTGACGCGATCAGG
ATTCTTGGAACTAATCTCTCCTACGACGAACTTAAGGCTCTTGCTGATACCTTTTCTCTTGATGAAAAC
GGGAACAAACTCAAGAAGGGAAAACACGGTATGCGGAATTTCATCATAAATAACGTTATTTCAAACA
AGAGATTCCATTACCTGATAAGATACGGAGATCCAGCCCATCTGCACGAAATCGCGAAAAACGAGGCT
GTTGTTAAATTCGTTTTGGGGAGAATCGCTGACATACAAAAAAAGCAGGGGCAAAACGGGAAGAACC
AGATCGACCGGTACTACGAAACCTGTATCGGTAAGGACAAAGGGAAGAGTGTGTCCGAAAAGGTTG
ACGCCCTTACAAAAATCATCACCGGTATGAACTATGACCAATTCGACAAGAAAAGAAGTGTTATAGAA
GATACGGGAAGAGAGAATGCGGAGCGGGAAAAATTTAAAAAGATCATATCGCTCTATCTGACCGTTAT
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GGACGCCCAGTTGTACAAAGAGAAAGGATACGACATAAACCTCAAGAAGCTCGAAGAGAAGGGTTT
TAGCTCTGTTACGAAACTTTGTGCGGGTATCGATGAAACCGCGCCTGACAAACGGAAAGACGTTGAA
AAGGAGATGGCAGAACGCGCTAAAGAGTCTATAGACAGTCTTGAGTCAGCAAATCCCAAGCTCTACG
CGAACTACATAAAATATTCTGACGAAAAAAAAGCTGAAGAATTTACCAGACAAATAAACAGAGAGAA
GGCTAAGACTGCGTTGAATGCCTATCTGCGGAACACTAAATGGAATGTCATAATTCGGGAAGACCTTC
TGCGGATCGACAATAAAACCTGCACCCTCTTT**AGAAATAAGGCTGTCCAC**CTGGAAGTTGCTCGCTA
TGTGCATGCGTATATTAACGACATTGCTGAGGTTAACAGCTACTTTCAGCTGTATCATTACATCATGCAG
AGGATTATTATGAACGAGCGCTACGAGAAGTCCTCCGGGAAGGTTTCAGAGTATTTTGACGCAGTCAA
CGATGAGAAAAAGTACAACGATCGGCTGCTGAAACTCCTGTGTGTCCCATTCGGGTATTGTATACCGC
GCTTCAAGAACCTCTCAATAGAGGCGCTCTTTGACCGCAACGAGGCCGCAAAGTTTGATAAAGAAAA
AAAGAAAGTTAGTGGGAATAGTGGCTCAGGACCAAAGAAGAAAAGAAAGGTTTGA

Amino acid sequences:

MSPKKKRKVEASIEKKKSFAKGMGVKSTLVSGSKVYMTTFAEGSDARLEKIVEGDSIRSVNEGEAFSAEM
ADKNAGYKIGNAKFSHPKGYAVVANNPLYTGPVQQDMLGLKETLEKRYFGESADGNDNICIQVIHNILDI
EKILAEYITNAAYAVNNISGLDKDIIGFGKFSTVYTYDEFKDPEHHRAAFNNNDKLINAIKAQYDEFDNFL
DNPRLGYFGQAFFSKEGRNYIINYGNECYDILALLSGL**RHWVVH**NNEEESRISRTWLYNLDKNLDNEYIS
TLNYLYDRITNELTNSFSKNSAANVNYIAETLGINPAEFAEQYFRFSIMKEQKNLGFNITKLREVMLDRKD
MSEIRKNHKVFDSIRTKVYTMMDFVIYRYYIEEDAKVAAANKSLPDNEKSLSEKDIFVINLRGSFNDDQK
DALYYDEANRIWRKLENIMHNIKEFRGNKTREYKKKDAPRLPRILPAGRDVSAFSKLMYALTMFLDGKEI
NDLLTTLINKFDNIQSFLKVMPLIGVNAKFVEEYAFFKDSAKIADELRLIKSFARMGEPIADARRAMYIDAI
RILGTNLSYDELKALADTFSLDENGNKLKKGKHGMRNFIINNVISNKRFHYLIRYGDPAHLHEIAKNEAVV
KFVLGRIADIQKKQGQNGKNQIDRYYETCIGKDKGKSVSEKVDALTKIITGMNYDQFDKKRSVIEDTGRE
NAEREKFKKIISLYLTVIYHILKNIVNINARYVIGFHCVERDAQLYKEKGYDINLKKLEEKGFSSVTKLCAG

IDETAPDKRKDVEKEMAERAKESIDSLESANPKLYANYIKYSDEKKAEEFTRQINREKAKTALNAYLRNT
KWNVIIREDLLRIDNKTCTLF**RNKAVH**LEVARYVHAYINDIAEVNSYFQLYHYIMQRIIMNERYEKSSGKV
SEYFDAVNDEKKYNDRLLKLLCVPFGYCIPRFKNLSIEALFDRNEAAKFDKEKKKVSGNSGSGPKKKRK
V*

** The highlighted sequences are SV40 NLS sequences.

*** The bold sequences are HEPN domain catalytic residues R-X4-H.

Sequence 2 (8 primer sequences used for the generation of the dCasRx):

Primer 1	ctttAGAaataaggctgtcGCTctggaagttgctcgc
Primer 2	GCaacaacccaatgCCGcagtccggacaacaatgcc
Primer 3	actgCGGcattgggttgttGCTaataacgaggaaga
Primer 4	AGCgacagccttattTCTaaagagggtgcaggtttta
Primer 5	ggcattgttgtccggactgGCTcattgggttgttGCT
Primer 6	AGCaaagagggtgcaggttttattgtcgatccgcaga
Primer 7	taaaacctgcaccctctttGCTaataaggctgtcGCT
Primer 8	AGCcagtccggacaacaatgccaaaatatcatagcac

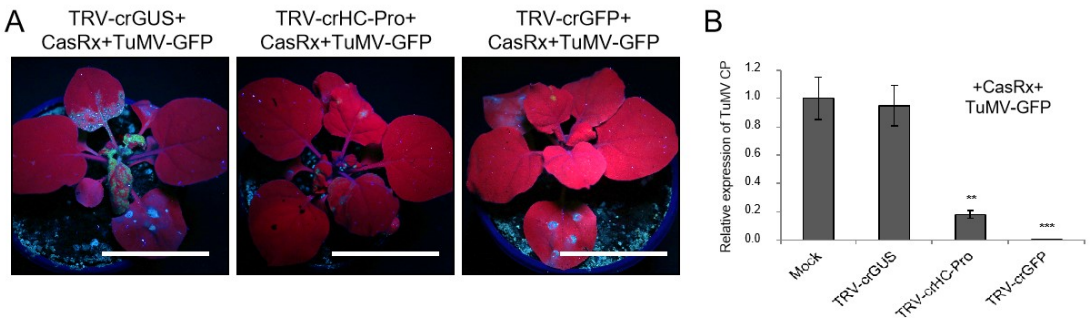

Fig. S1 The CasRx-mediated suppression of TuMV infection by sequential inoculations of programmable CasRx systems prior to TuMV infectious clone. A GFP fluorescence in the systemic leaves infected with sequential inoculations of TRV vector-based programmable CasRx systems prior to the corresponding TuMV infectious clone. *Agrobacterium* cultures harboring the following constructs to express CasRx and TRV1, together with TRV2-PEBV:crGUS (TRV-crGUS), or TRV2-PEBV:crHC-Pro (TRV-crHC-Pro), or TRV2-PEBV:crGFP (TRV-crGFP) were infiltrated onto *N. benthamiana* leaves, and the infiltrated leaves at 3 dpi were inoculated with TuMV infectious clone. The TuMV-infected plants at 7 dpi were photographed under UV light. Scale bar = 4 cm. **B** Relative expression level of the TuMV CP in the systemic leaves indicated in (A). The relative TuMV CP RNA levels were analyzed by RT-qPCR, and *NbActin* served as an internal reference gene. These data were analyzed using two-sided Student's t-test, $^{**}P < 0.01$, $^{***}P < 0.001$.