CRISPR-Cas12a genome editing at the whole-plant level using two compatible RNA virus vectors

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Abstract

The use of viral vectors that can replicate and move systemically through the host plant to deliver bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR) components enables genome editing at the whole-plant level and avoids the requirement for labor-intensive stable transformation. However, this approach usually relies on previously transformed plants that stably express a CRISPR-associated (Cas) nuclease. Here we describe successful DNA-free genome editing of *Nicotiana benthamiana* using two compatible RNA virus vectors, derived from tobacco etch virus (TEV; genus *Potyvirus*) and potato virus X (PVX; genus *Potexvirus*), which replicate in the same cells. The TEV and PVX vectors respectively express a Cas12a nuclease and the corresponding guide RNA. This novel two-virus vector system improves the toolbox for transformation-free virus-induced genome editing in plants and will advance efforts to breed more nutritious, resistant, and productive crops.

Introduction

Systems derived from bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins (Cong *et al.*, 2013) have revolutionized biotechnology. In plants, CRISPR-Cas holds great promise for unprecedented genome engineering of both model species and crops (Nekrasov *et al.*, 2013; Zhang *et al.*, 2016; Li *et al.*, 2017; Zhu *et al.*, 2020; Huang and Puchta, 2021). Most common CRISPR/Cas arrangements include a Cas endonuclease, such as *Streptococcus pyogenes* SpCas9, and a single-guide RNA

(sgRNA), which specifically directs the nuclease to a sequence of interest in the genome. As in other taxonomic groups (Platt et al., 2014; Senís et al., 2014; Lau and Suh, 2017; Xu et al., 2019), virus-derived vectors have been reported as a powerful alternative to express the CRISPR/Cas components at the whole-plant level avoiding the labor-intensive and time-consuming tissue culture approaches required for stable transformation. These strategies are commonly termed as virus-induced genome editing (VIGE) and have focused on the delivery of one or more sgRNAs using RNA or DNA virus vectors in transgenic plants that stably express the Cas nuclease (Ali *et al.*, 2015; Yin *et al.*, 2015; Cody *et al.*, 2017; Ali *et al.*, 2018; Hu *et al.*, 2019; Jiang *et al.*, 2019; Ellison *et al.*, 2020; Lei *et al.*, 2021).

Expression of a Cas nuclease using a plant virus-derived vector able to move systemically through a plant was long considered unachievable due to cargo constraints. However, the innovative work by Ma et al. (2020) demonstrated efficient genome editing by delivering both the sgRNA and SpCas9 at the whole-plant level using a vector derived from sonchus yellow net virus (SYNV; family *Rhabdoviridae*). Despite this unprecedented achievement, additional virus-based systems for the co-expression of Cas nucleases and sgRNAs at the whole-plant level are still required to improve the current toolbox for crop engineering. Notably, each viral vector has its own unique properties, particularly a specific host range.

To expand the virus-based tools for tissue culture-free genome editing in plants, we co-expressed the Cas nuclease and the guide RNA using two compatible viral vectors that replicate in the same cells and coordinately move systemically through the whole-plant. We chose a potyvirus vector to express the Cas nuclease. Potyviruses (genus *Potyvirus*) are the largest group of plus-strand RNA viruses, with more than 200 currently known species that collectively can infect a large range of host plants (Wylie *et al.*, 2017). We also focused on a Cas12a (formerly Cpf1) nuclease, which is a component of a class 2 type V CRISPR system, isolated from *Lachnospiraceae bacterium* ND2006 (LbCas12a) (Zetsche et al., 2015). Genome editing using LbCas12a has been demonstrated in plants (Endo et al., 2016; Tang et al., 2017; Wang et al., 2017; Xu et al., 2017), and the complementary DNA (cDNA) corresponding to this nuclease is smaller than that of SpCas9. We also co-expressed the guide RNA using a recently described potato virus X (PVX, genus *Potexvirus*, family *Virgaviridae*) vector, which efficiently induces hereditable gene editing in *Nicotiana benthamiana* plants that stably express SpCas9 (Uranga *et al.*, 2021). Our results demonstrated efficient DNA-free *N. benthamiana* genome editing using the two compatible RNA virus vectors.

Methods

Viral vectors

Guide RNAs to target *N. benthamiana Flowering locus T* (*NbFT*; SolGenomics Niben101Scf01519g10008.1) and *Xylosyl transferase 1* (*NbXT1*; Niben101Scf04205g03008.1) were selected using the CRISPR-P online tool as described by Bernabé-Orts et al. (2019) (Table S1). Nucleotide (nt) sequences of recombinant viral clones are shown in Figs. S1 and S2. These clones were built using the primers shown in Tables S2 and S3.

Plant inoculation

N. benthamiana wild-type and transformed plants expressing tobacco etch virus (TEV, genus *Potyvirus*) nuclear inclusion *b* (NIb) protein (Martí *et al.*, 2020), were grown at 25ºC under a 12 h/12 h day/night photoperiod. Plants that were 4- to 6-weeks-old were agroinoculated as previously described (Bedoya *et al.*, 2010; Uranga *et al.*, 2021). Tissue samples (approximately 100 mg) from the first symptomatic upper non-inoculated leaf were collected at different days post inoculation (dpi), as indicated, for virus progeny and plant genome-editing analyses.

Reverse transcription (RT)-polymerase chain reaction (PCR) analysis of viral progeny

RNA was purified from leaf samples using silica gel columns (Uranga *et al.*, 2021). cDNA was synthesized using RevertAid reverse transcriptase (Thermo Scientific) and primer D179 (Table S4). PCR with *Thermus thermophilus* DNA polymerase (Biotools) was used to amplify the TEV coat protein (CP) cistron (primers D178 and D211; Table S4) or a fragment of the LbCas12a open reading frame (ORF) (primers D3604 and D3605; Table S4). PCR products were separated by electrophoresis in 1% agarose gels followed by staining with ethidium bromide.

Analysis of *N. benthamiana* genome editing

DNA from leaf samples was purified using silica gel columns (Uranga *et al.*, 2021). *N. benthamiana* genome fragments were amplified by PCR using high-fidelity Phusion DNA polymerase (Thermo Scientific) (Table S5). PCR products were separated by agarose gel electrophoresis, purified from the gel, and subjected to Sanger sequencing (Table S5). The presence of sequence modifications was analyzed using the inference of CRISPR edits (ICE) software (http://www.synthego.com/products/bioinformatics/crispr-analysis).

Results

A dual virus-based vector system to co-express Cas nucleases and guide RNAs in plants

Initially, we built a TEV recombinant clone in which the cDNA of a human codon-optimized LbCas12a replaced that of the viral NIb protein (TEVΔN::LbCas12a). Our previous work showed that this vector could express large exogenous sequences, such as a whole bacterial metabolic pathway to biosynthesize lycopene or the saffron carotenoid cleavage dioxygenase (Majer *et al.*, 2017; Martí *et al.*, 2020). However, since the virus lacks the viral RNA-dependent RNA polymerase NIb, it replicates only in plants that express this protein (Majer *et al.*, 2017). In our recombinant clone, the sequence coding for LbCas12a replaced most of the NIb cistron and was flanked by the native nuclear inclusion *a* protease (NIaPro) cleavage sites that mediate the release of the nuclease from the viral polyprotein (Fig. 1A and Fig. S1).

Next, we built a recombinant version of PVX in which the LbCas12a CRISPR RNA (crRNA) was expressed under the control of the viral CP promoter, and the 29 initial codons of PVX CP were deleted to improve the stability of the recombinant clone (Dickmeis et al., 2014). The viral CP was expressed from a heterologous promoter derived from that of the CP of bamboo mosaic virus (BaMV; genus *Potexvirus*). Based on previous work by Bernabé-Orts et al. (2019) assessing the efficiency of the Cas12a-mediated gene editing of several *N. benthamiana* loci, *NbFT* was selected as the target gene (PVX::crFT) (Fig. 1B and Fig. S2). The 65-nt crRNA was cloned downstream of the PVX CP promoter and consisted of a 23-nt protospacer sequence specific to the target gene flanked on both 5' and 3' ends by a conserved 21-nt scaffold, also known as a direct repeat.

N. benthamiana plants constitutively expressing TEV NIb under the control of cauliflower mosaic virus (CaMV) 35S promoter and terminator (Martí *et al.*, 2020) were co-inoculated with a 1:1 mix of two cultures of *Agrobacterium tumefaciens* transformed with plasmids harboring TEVΔNIb::LbCas12a and PVX::crFT constructs. Controls included inoculation of TEVΔNIb::LbCas12a alone and inoculation of TEVΔNIb::crtB, which allows visual tracking of virus systemic movement due to the yellow pigmentation of infected tissue induced by *Pantoea ananatis* phytoene synthase (crtB) (Majer et al., 2017). At 7 dpi, typical symptoms of TEV infection emerged in the upper non-inoculated leaves of all plants. Notably, at 14 dpi irregular chlorotic spots appeared in the leaves of plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT, but not in those inoculated with TEVΔNIb::LbCas12a alone (Fig. S3). PVX infection is characterized by the appearance of vein banding, ring spots

and leaf atrophy (Loebenstein and Gaba, 2012). Therefore, the observed phenotypic alteration could have been due to LbCas12a-mediated editing of *NbFT* or an effect of the coinfection. Samples from the first systemically infected upper leaf were collected at 7 and 14 dpi, and TEVΔNIb progeny were studied by RT-PCR analysis (Fig. 1C). An 804-bp specific region of the TEV genome corresponding to the CP cistron was amplified in all virus-inoculated plants, confirming the presence of the virus (Fig. 1C, top panel). However, for viral vectors carrying big cargos, genomes larger than wild type are likely to recombine to smaller sizes, thus triggering the loss of heterologous genes (Gilbertson et al., 2003). An additional RT-PCR analysis was performed, and a 600-bp cDNA corresponding to a fragment of the LbCas12a ORF was exclusively amplified from plants inoculated with TEVΔNIb::LbCas12a alone or co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT, regardless of sampling time (Fig. 1C, bottom panel). These results suggested that the LbCas12a nuclease was expressed from the onset of viral infection and throughout the rest of the experiment.

Next, DNA was purified from leaf samples collected at 7 and 14 dpi, and a 550-bp fragment of the *NbFT* gene covering the LbCas12a target site was amplified by PCR. Sanger sequencing of the PCR products and ICE analysis revealed robust gene editing at 14 dpi in plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT, reaching an indel percentage of up to 75% (Fig. 1D and Fig. S4). No significant differences were observed at 21 and 28 dpi. The indel distribution was consistent with the deletion-enriched mutagenesis profile characteristic of Cas12a activity, being mainly 5- to 10-bp deletions (Bernabé-Orts et al., 2019). These results indicate that the simultaneous delivery of CRISPR-Cas12a components through two compatible viral vectors (i.e., TEVΔNIb and PVX) allows highly efficient, DNA-free targeted mutagenesis in *N. benthamiana*.

Multiplex genome editing using the dual virus-based vector system in plants

A key advantage of CRISPR-Cas genome editing is the capacity to target several loci at once by the simultaneous expression of several guide RNAs (i.e., multiplexing). Based on our observations in Cas9-expressing plants (Uranga *et al.*, 2021), we wondered whether the PVX vector could allow the delivery of multiple, functional crRNAs for Cas12a-mediated editing. To investigate this, we selected *Xylosyl transferase 1* (*NbXT1*) as the second target gene. As Cas12a can self-process crRNAs due to its RNase III activity, *NbXT1* and *NbFT* crRNAs were arranged in tandem under the control of the same CP promoter, thus creating the PVX::crXT1:crFT construct (Fig. 1E). *N. benthamiana* plants constitutively expressing TEV

NIb were co-inoculated with a 1:1 mix of two *A. tumefaciens* cultures carrying TEVΔNIb::LbCas12a and PVX::crXT1:crFT. *A. tumefaciens* transformed with TEV::crtB was used as a control in this assay. The first systemically infected leaf was sampled at 14, 21, and 28 dpi, following extraction of genomic DNA and PCR amplification of the target sites. ICE analysis revealed efficient gene editing on both *NbXT1* and *NbFT*, with average indel percentages ranging from 76% to 88%, which was maintained regardless of the sampling time (Fig. 1F). In addition, the absence of statistically relevant differences in gene editing among *NbXT1* and *NbFT* suggested that LbCas12a can efficiently self-process tandemly arrayed crRNAs. Time-course comparison with the single-crRNA construct, in the case of *NbFT*, also revealed that multiplexing does not affect the editing efficiency, since the mutation rates achieved with both strategies were similar (Fig. 1D and F).

Dual vector CRISPR-Cas genome editing in wild-type plants

The fact that TEVΔNIb infectivity depends on the supplementation of viral NIb from a transgene may be perceived as a limitation of this genome-editing system, as the approach is still bound to a previously transformed plant. An alternative strategy for supplying NIb activity consists of the co-inoculation of TEVΔNIb with a recombinant PVX expressing NIb (Bedoya et al., 2010). We wondered whether a single PVX vector could: (i) provide NIb activity for the systemic movement of TEVΔNIb; and (ii) deliver the crRNA for LbCas12a-mediated gene editing. Thus, the coding sequence of the TEV NIb cistron plus an additional amino-terminal Met was inserted within the expression cassette in PVX. *NbFT*-specific crRNA was added downstream of NIb without any linker sequence, so that both NIb and crFT expression were under the control of the PVX CP promoter (PVX::NIb:crFT) (Fig. 2A). Wild-type *N. benthamiana* plants were co-inoculated with a 1:1 mix of two cultures of *A. tumefaciens* carrying TEVΔNIb::LbCas12a and PVX::NIb:crFT. *A. tumefaciens* transformed with TEV::crtB was again used as a control. At 7 dpi, the apical leaves of the co-inoculated *N. benthamiana* plants became symptomatic (i.e., leaf curling). At 14 dpi, necrotic spotting and interveinal mottling were observed in systemic leaves, which became more noticeable over time (Fig. S5). Samples from the first systemically infected upper leaf were collected at 14 dpi and the 550-bp fragment of the *NbFT* gene covering the LbCas12a target site was amplified by PCR. ICE analysis of the PCR products exhibited 20% indels in plants co-inoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT. These results indicate that a single PVX vector can

supply the viral NIb activity that allows TEVΔNIb to systemically spread in wild-type *N. benthamiana*, as well as to perform crRNA delivery for LbCas12a-mediated genome editing.

Discussion

In this study we describe the engineering of a dual RNA-virus system for the delivery of CRISPR-Cas12a components for genome editing in plants. The system consists of two compatible plus-strand RNA viruses, specifically the potyvirus TEV and the potexvirus PVX, to express the Cas nuclease and the guide RNA, respectively. Notably, the TEV vector (TEVΔNIb) contains the deletion of the RNA-dependent RNA polymerase (NIb) in order to accommodate the large ORF of the Cas nuclease. Our previous efforts to express a functional Cas nuclease using a full-length potyvirus vector in plants have been unsuccessful. However, recent works have reported successful Cas9 expression and DNA-free genome editing using single SYMV (Ma et al., 2020) and PVX (Ariga *et al.*, 2020) vectors in *N. benthamiana*. These contrasting results emphasize the diverse properties of vectors derived from viruses belonging to different genera and families, and the necessity for a large spectrum of molecular tools, operating in a wide range of host species, to tackle challenging VIGE goals in crop plants. Notably, SYNV is a minus-strand RNA virus the inoculation of which entails some complexity (Peng *et al.*, 2021) and, as with each plant virus, it exhibits a particular host range (Jackson and Christie, 1977).

In this work, we aimed to express a Cas nuclease using a potyvirus vector for DNA-free plant-genome editing. The genus *Potyvirus* is the largest among the plant RNA viruses, and cromprises more than 200 species that infect a wide range of host plants from many different botanical families (Wylie *et al.*, 2017). Therefore, it offers a wealth of genetic resources for VIGE. Although we were unable to successfully express the Cas nuclease using a full-length potyvirus vector, we demonstrated that TEVΔNIb allows the transient expression of LbCas12a, and that PVX can perform both single or multiple crRNA delivery as well as providing the NIb activity. Our two-virus delivery system resulted in efficient DNA-free targeted editing both in NIb-expressing and wild-type *N. benthamiana* plants, reaching indel percentages of up to 80% and 20%, respectively (Figs. 1 and 2). This dual vector system not only incorporates the enormous genetic resources of potyviruses for VIGE, but also demonstrates that compatible RNA virus vectors can be used to simultaneously deliver several CRISPR-Cas components. This could be useful for more sophisticated arrangements in DNA-free plant-genome editing and gene-expression regulation studies. Recently, we developed a PVX vector to efficiently

express multiplex SpCas9 sgRNAs at the whole-plant level. Current results with LbCas12a confirm that PVX can be easily engineered for the simultaneous delivery of guide RNAs regardless of the nature of the Cas nuclease, which highlights its usefulness in a variety of multiplexing approaches.

In contrast to previous reports of DNA-free VIGE that used SpCas9 (Ariga *et al.*, 2020; Ma *et al.*, 2020), we selected Cas12a. This was due to the following unique features of the nuclease (Zaidi et al., 2017): (i) the cleavage of target DNA is directed by a single crRNA shorter than that of SpCas9 sgRNA; (ii) the protospacer adjacent motif (PAM) is T-rich (5'-TTTN-3'); (iii) DNA cleavage results in cohesive ends with 4- or 5-nt overhangs, which might facilitate homology-directed repair (HDR); (iv) it exhibits RNase III activity useful to facilitate multiplex gene editing; and (v) it is smaller than SpCas9 (3.8 kb vs 4.2 kb), which is important in terms of viral delivery. Moreover, the unique characteristics of the CRISPR-Cas12a system, unlike that of Cas9, such as the recognition of a T-rich PAM and the induction of staggered ends that facilitate homologous recombination, limit the range of target sequences and thus reduce off-target activity (Zaidi et al., 2017). Cas12a orthologs from *Francisella novicida* U112 (FnCas12a), *Acidaminococcus* sp. BV3L6 (AsCas12a) and LbCas12a were first experimentally validated in mammalian cells (Kim et al., 2016, 2017a; Zetsche et al., 2017). In plants, targeted mutagenesis was achieved in rice and tobacco using any of the orthologs (Endo et al., 2016; Tang et al., 2017; Wang et al., 2017; Xu et al., 2017). A DNA-free approach based on the delivery of AsCas12a or LbCas12a loaded with crRNA was also validated in wild tobacco and soy-bean protoplasts (Kim et al., 2017b). Here we focused on LbCas12a, since previous works reported that this nuclease possesses higher efficiency than FnCas12a or AsCas12a (Tang et al., 2017; Bernabé-Orts et al., 2019), and is also effective for plant-genome editing when virally delivered.

In conclusion, our dual RNA-virus-based system broadens the current toolbox for DNA-free VIGE and will contribute to applications in plant functional genomics and crop improvement.

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Supplementary Material

Figure S1. Full sequence of wild-type tobacco etch virus (TEV, Genbank accession number DQ986288), the defective viral vector TEVΔNIb and their derived recombinant viruses, TEV::crtB, TEVΔNIb::crtB and TEVΔNIb::LbCas12a.

Figure S2. Full sequence of wild-type potato virus X (PVX; GenBank accession number MT799816) and its derived recombinant viruses PVX::crFT and PVX::NIb:crFT.

Figure S3. 35S::NIb *N. benthamiana* plants constitutively expressing TEV NIb and representative leaves from these plants at 14 dpi inoculated with TEVΔNIb::LbCas12a or co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT.

Figure S4. Example of a sequence electropherogram and mutagenesis profile from 35S::NIb N. benthamiana plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT.

Figure S5. Representative leaves from wild-type *N. benthamiana* plants at 14 dpi inoculated with TEVΔNIb::LbCas12a alone, PVX::NIb:crFT alone, or coinoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT.

Table S1. *N. benthamiana* genes targeted by the CRISPR-Cas12a system. PAMs are highlighted with a grey background.

Table S2. Primers used for the construction of recombinant viruses.

Table S3. Primer combinations used for the construction of recombinant viruses.

Table S4. Primers used for TEVΔNIb diagnosis by RT-PCR.

Table S5. Primers used for Cas12a-crRNA gene editing analysis.

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Figures

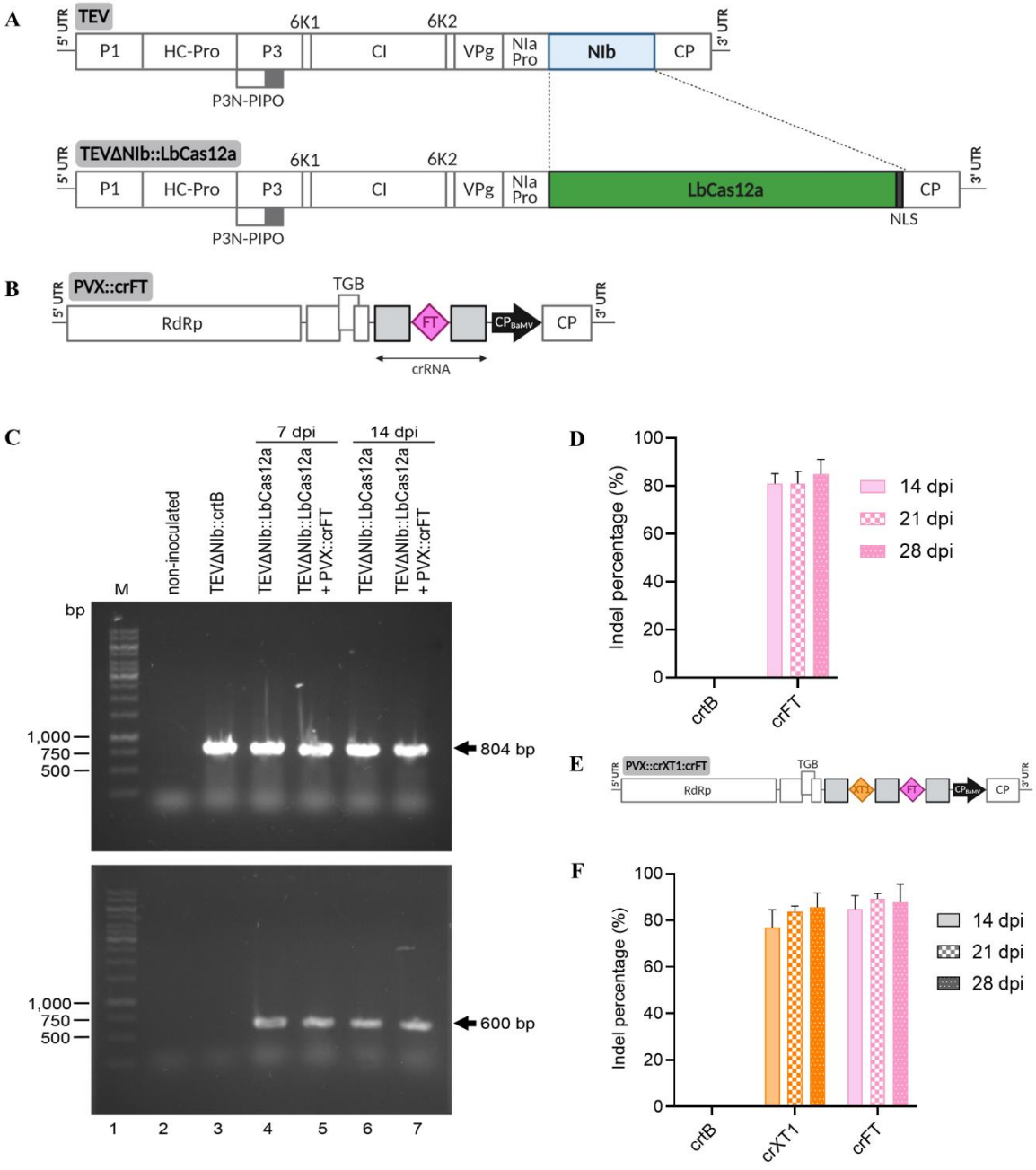

FIG. 1. DNA-free gene editing in *N. benthamiana* based on virally delivered LbCas12a and crRNA. **(A)** Schematic representation of recombinant virus TEVΔNIb::LbCas12a. Cas12a ORF from *Lachnospiraceae bacterium* ND2006 (LbCas12a), containing a carboxy-terminal nucleoplasmin nuclear localization signal (NLS, grey box), replaces the TEV NIb cistron (light blue box) and is flanked by proteolytic processing sites of viral NIaPro. TEV cistrons P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, VPg, NIaPro, NIb, and CP are represented by boxes. 5' and 3' untranslated regions (UTRs) are represented by lines. **(B)** Schematic representation of

recombinant clone PVX::crFT. RNA-dependent RNA polymerase (RdRp), triple gene block (TGB), and coat protein (CP) are represented by open boxes. Heterologous *Bamboo mosaic virus* CP promoter (CP_{BaMV}) is represented by a black arrow. 5' and 3' UTRs are represented by black lines. crRNA consists of a gene-specific 23-nt protospacer (pink diamond) and conserved 21-nt direct repeats (grey boxes). CP_{BaMV}, protospacer and scaffold are not shown at scale. **(C)** RT-PCR analysis of TEVΔNIb progeny at 7 and 14 dpi in 35S::NIb *N. benthamiana* plants inoculated with TEVΔNIb::LbCas12a alone or co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT. Amplification products were separated by electrophoresis in an agarose gel stained with ethidium bromide. Lane 1, 1-kb ladder DNA marker with the length of some components (in bp) indicated on the left; lane 2, non-inoculated plant; lanes 3 to 7, plants inoculated with TEVΔNIb::crtB (lane 3) or TEVΔNIb::LbCas12a (lanes 4 and 6), and co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT (lanes 5 and 7). The amplification products corresponding to cDNA regions of TEV CP (804 bp) (top) or LbCas12a (600 bp) (bottom) are indicated by arrows. **(D)** ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT at the indicated dpi (n=6). TEVΔNIb::crtB was used as a negative control. (**E**) Schematic representation of recombinant clone PVX::crXT1:crFT. Protospacers for NbXT1 and NbFT are represented by orange and pink diamonds, respectively. Other details are indicated above. (**F**) ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants (n=6) co-inoculated with TEVΔNIb::LbCas12a and PVX::crXT1:crFT at the indicated dpi. Columns and error bars represent average indels (%) and standard deviation, respectively.

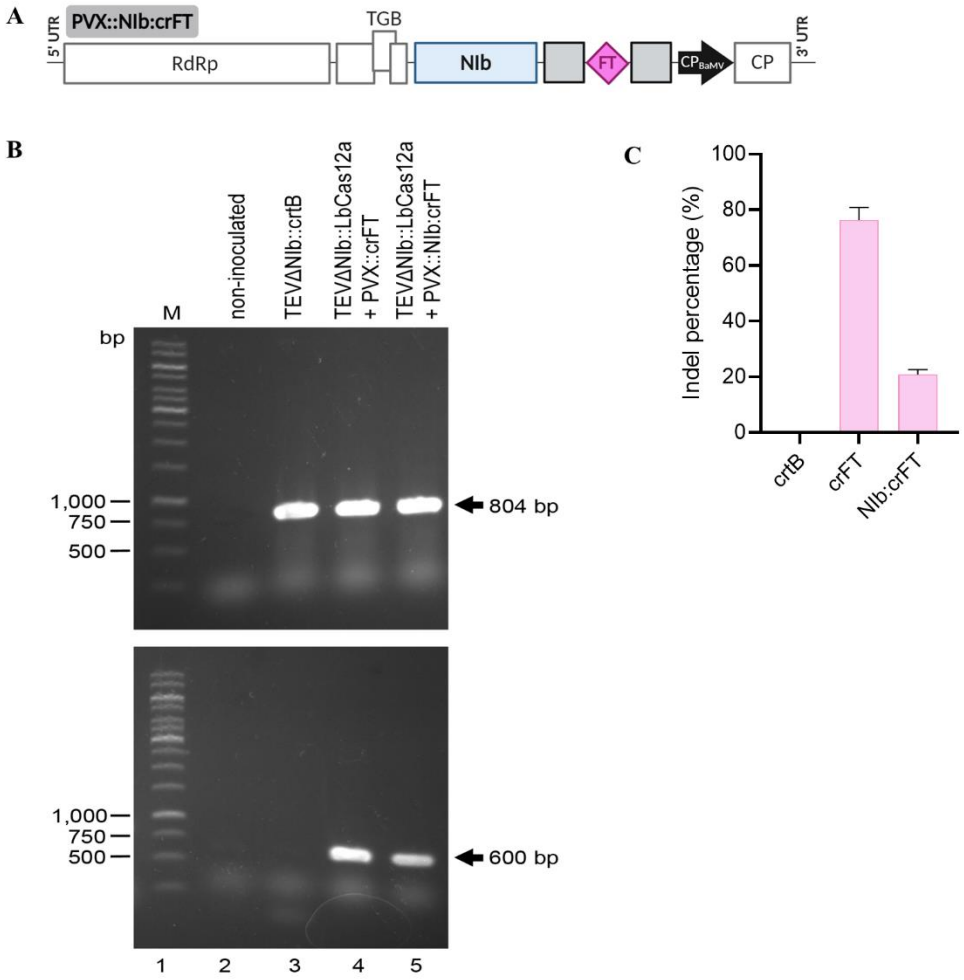

FIG. 2. Engineering of a single PVX vector for complementation of defective TEVΔNIb and expression of LbCas12a crRNA. **(A)** Schematic representation of recombinant virus PVX::NIb:crFT. TEV NIb cistron is represented by a light blue box. Other details are as described in the legend to Fig. 1. **(B)** RT-PCR analysis of TEVΔNIb progeny at 14 dpi in wild-type *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT or with TEVΔNIb::LbCas12a and PVX::NIb:crFT. Amplification products were separated by electrophoresis in an agarose gel that was stained with ethidium bromide. Lane 1, DNA marker ladder with the length of some components (in bp) indicated on the left; lane 2, non-inoculated plant; lane 3 to 5, plants inoculated with TEVΔNIb::crtB (lane 3) and co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT (lane 4) or with TEVΔNIb::LbCas12a and PVX::NIb:crFT (lane 5). The amplification products corresponding to cDNA regions of TEV CP (804 bp) (top) or LbCas12a (600 bp) (bottom) are indicated by arrows. (c) ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT at 14 dpi (n=6). TEVΔNIb::crtB was used as a

453 negative control. Columns and error bars represent average indels (%) and standard deviation,
454 respectively.

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SUPPLEMENTAL DATA

CRISPR-Cas12a genome editing at the whole-plant level using two compatible RNA virus vectors

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Figure S1. Full sequence of the wild-type tobacco etch virus (TEV), the defective viral vector TEVΔNIb and their derived recombinant viruses, TEVΔNIb::crtB and TEVΔNIb::LbCas12a. TEV-wt sequence corresponds to Genbank accession number DQ986288 including two silent mutations (G273A and A1119G, in red). Limits between TEV cistrons are marked on blue background. cDNAs corresponding to *P. ananatis* **phytoene synthase** (crtB) and **Cas12a from *Lachnospiraceae bacterium* ND2006** (LbCas12a) are on yellow and dark green backgrounds, respectively. Nucleoplasmin nuclear localization signal (NLS) and human influenza hemagglutinin (3xHA) tag are underlined and dotted, respectively. In the inserted cDNAs, sequences corresponding to **native** and **artificial** TEV NIaPro cleavage sites are in black or blue, respectively.

>TEV

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>TEVΔNIb

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>TEVΔNIb::LbCas12a (insert between positions 6981-6982 of TEVΔNIb)

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773 GCCCTGGAGGACCTGAACTCTGGCTTTAAGAATAGCCGCGTGAAGGTGGAGAAGCAGGTGTATCAGAAGTTCGAG
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777 GATTCCAAGAAGTTCATCAGCTCCTTTGACAGGATCATGTACGTGCCCGAGGAGGATCTGTTCGAGTTTGCCCTG
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785 CAGACCAGCGTGAAGCACAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAAAAAAGAAAAAGGGATCCTAC
786 CCATACGATGTTCCAGATTACGCTTATCCCTACGACGTGCCTGATTATGCATACCCATATGATGTCCCCGACTAT
787 GCCACGACTGAGAATCTTTATTTTCAG
788
789 **Figure S2.** Full sequence of wild-type potato virus X (PVX; GenBank accession number
790 MT799816) and its derived recombinant viruses PVX::crFT and PVX::NIb:crFT. In
791 recombinant PVX clones, heterologous sequences are transcribed from coat protein (CP)
792 promoter and a deleted version of PVX CP, lacking the 29 initial codons, is transcribed from
793 an heterologous promoter derived from *Bamboo mosaic virus* (BaMV) CP (Dickmeis et al.,
794 2014). **PVX CP promoter** with a ATG-AGG mutation to abolish start codon is underlined.
795 **BaMV CP promoter** is in blue. crRNA sequences corresponding to *NbFT* and *NbXT1* are on
796 pink and red background, respectively, and **protospacer** region is underlined (dotted). cDNA
797 corresponding to **TEV nuclear inclusion *b* (NIb) cistron** is on dark blue background.
798
799 **>PVX (MT799816)**
800 GAAAACTAAACCATACACCACCAACACAACCAAACCCACCACGCCCAATTGTTACACACCCGCTTGAAAAAGAAA
801 GTTTAACAAATGGCCAAGGTGCGCGAGGTTTACCAATCTTTTACAGACTCCACCACAAAAACTCTCATCCAAGAT
802 GAGGCTTATAGAAACATTCGCCCCATCATGGAAAAACACAAACTAGCTAACCCTTACGCTCAAACGGTTGAAGCG
803 GCTAATGATCTAGAGGGGTTCGGCATAGCCACCAATCCCTATAGCATTGAATTGCATACACATGCAGCCGCTAAG
804 ACCATAGAGAATAAACTTCTAGAGGTGCTTGGTTCCATCCTACCACAAGAACCTGTTACATTTATGTTTCTTAAA
805 CCCAGAAAGCTAAACTACATGAGAAGAAACCCGCGGATCAAGGACATTTTCCAAAATGTTGCCATTGAACCAAGA
806 GACGTAGCCAGGTACCCCAAGGAAACAATAATTGACAAACTCACAGAGATCACAACGGAAACAGCATACATTAGT
807 GACACTCTGCACTTCTTGGATCCGAGCTACATAGTGGAGACATTCCAAAACTGCCCAAAATTGCAAACATTGTAT

808 GCGACCTTAGTTCTCCCCGTTGAGGCAGCCTTTAAAATGGAAAGCACTCACCCGAACATATACAGCCTCAAATAC
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868 TCCACAGCTTTCTACAACGCTATCACCAGGTCAAAGGGATTGACATATGTCCGCGCAGGGCCATAGGCTGACCGC

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TAGAAATAGCTTACCCCACGTCGGTGACAACATTCACAGCTTGCCACACGGAGGAGCTTACAGAGACGGCACCAA
AGCAATCTTGTACAACTCCCCAAATCTAGGGTCACGAGTGAGTCTACACAACGGAAAGAACGCAGCATTTGCTGC
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CAATCATAGCAGTCATTAGCACTTCCTTAGTGAGGACTGAACCTTGTGTCATCAAGATTACTGGGGAATCAATCA
CAGTGTTGGCTTGCAAACTAGATGCAGAAACCATAAGGGCCATTGCCGATCTCAAGCCACTCTCCGTTGAACGGT
TAAGTTTCCATTGATACTCGAAAGATGTCAGCACCAGCTAGCACAACACAGCCCATAGGGTCAACTACCTCAACT
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AGTCCACCTGCTAACTGGCAAGCACAAGGTTTCAAGCCTGAGCACAAATTCGCTGCATTCGACTTCTTCAATGGA
GTCACCAACCCAGCTGCCATCATGCCCAAAGAGGGGCTCATCCGGCCACCGTCTGAAGCTGAAATGAATGCTGCC
CAAACTGCTGCCTTTGTGAAGATTACAAAGGCCAGGGCACAATCCAACGACTTTGCCAGCCTAGATGCAGCTGTC
ACTCGAGGTCGTATCACTGGAACAACAACCGCTGAGGCTGTTGTCACTCTACCACCACCATAACTACGTCTACAT
AACCGACGCCTACCCCAGTTTCATAGTATTTTCTGGTTTGATTGTATGAATAATATAAATAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

>PVX::crFT (insert between A5585 and T5737 of PVX-wt)

AGGGCCATTGCCGATCTCAAGCCACTCTCCGTTGAACGGTTAAGTTTCCATTGATACTCGAAAGAGGTCAGCACC
AGCTAGCATAATTTCTACTAAGTGTAGATCAAGATCTATTGGCCTAAGAGTTTAATTTCTACTAAGTGTAGATTA
GGGTTTGTTAAGTTTCCCTTTTTACTCGAAAGATG

>PVX::crXT1:crTFL1:crFT (insert between A5585 and T5737 of PVX-wt)

AGGGCCATTGCCGATCTCAAGCCACTCTCCGTTGAACGGTTAAGTTTCCATTGATACTCGAAAGAGGTCAGCACC
AGCTAGCATAATTTCTACTAAGTGTAGATTATGTAGGTGTATTTGGAATTCTTAATTTCTACTAAGTGTAGATCA
AGATCTATTGGCCTAAGAGTTTAATTTCTACTAAGTGTAGATTAGGGTTTGTTAAGTTTCCCTTTTTACTCGAAA
GATG

>PVX:NIb:crFT (insert between A5585 and T5737 of PVX-wt)

AGGGCCATTGCCGATCTCAAGCCACTCTCCGTTGAACGGTTAAGTTTCCATTGATACTCGAAAGAGGTCAGCACC
AGCTAGCAATGGGGGAGAAGAGGAAATGGGTCGTGGAAGCACTGTCAGGGAACTTGAGGCCAGTGGCTGAGTGTC
CCAGTCAGTTAGTCACAAAGCATGTGGTTAAAGGAAAGTGTCCCCTCTTTGAGCTCTACTTGCAGTTGAATCCAG
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TCATCACAAAGCTCAAGGCGTTAGGATTCCCAACTGTGAACTACATCACTGACCCAGAGGAAATTTTTAGTGCAT
TGAATATGAAAGCAGCTATGGGAGCACTATACAAAGGCAAGAAGAAAGAAGCTCTCAGCGAGCTCACACTAGATG
AGCAGGAGGCAATGCTCAAAGCAAGTTGCCTGCGACTGTATACGGGAAAGCTGGGAATTTGGAATGGCTCATTGA
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TGGAGGAATGGGATATTGGTGAGCAAATGCTGCGAAATTTGTACACTGAGATAGTGTATACACCAATCCTCACAC
CGGATGGTACTATCATTAAGAAGCATAAAGGCAACAATAGCGGGCAACCTTCAACAGTGGTGGACAACACACTCA
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AGAGGGATGGCATGTATATACCAAAGCTAGAAGAAGAAAGGATTGTTTCTATTTTGGAATGGGACAGATCCAAAG
AGCCGTCACATAGGCTTGAAGCCATCTGTGCATCAATGATCGAAGCATGGGGTTATGACAAGCTGGTTGAAGAAA
TCCGCAATTTCTATGCATGGGTTTTGGAACAAGCGCCGTATTCACAGCTTGCAGAAGAAGGAAAGGCGCCATATC
TGGCTGAGACTGCGCTTAAGTTTTTGTACACATCTCAGCACGGAACAAACTCTGAGATAGAAGAGTATTTAAAAG
TGTTGTATGATTACGATATTCCAACGACTGAGAATCTTTATTTTCAGTAATAATTTCTACTAAGTGTAGATCAAG
ATCTATTGGCCTAAGAGTTTAATTTCTACTAAGTGTAGATTAGGGTTTGTTAAGTTTCCCTTTTTACTCGAAAGA
TG

Figure S3. 35S::NIb *N. benthamiana* plants and representative leaves from these plants at 14 dpi inoculated with TEVΔNIb::LbCas12a (left) or co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT (right). Chlorotic spots in leaves from co-inoculated plants are indicated with red arrows.

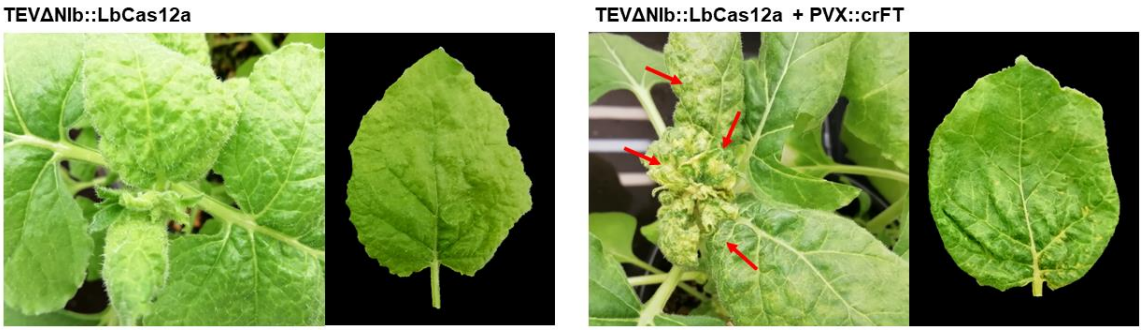
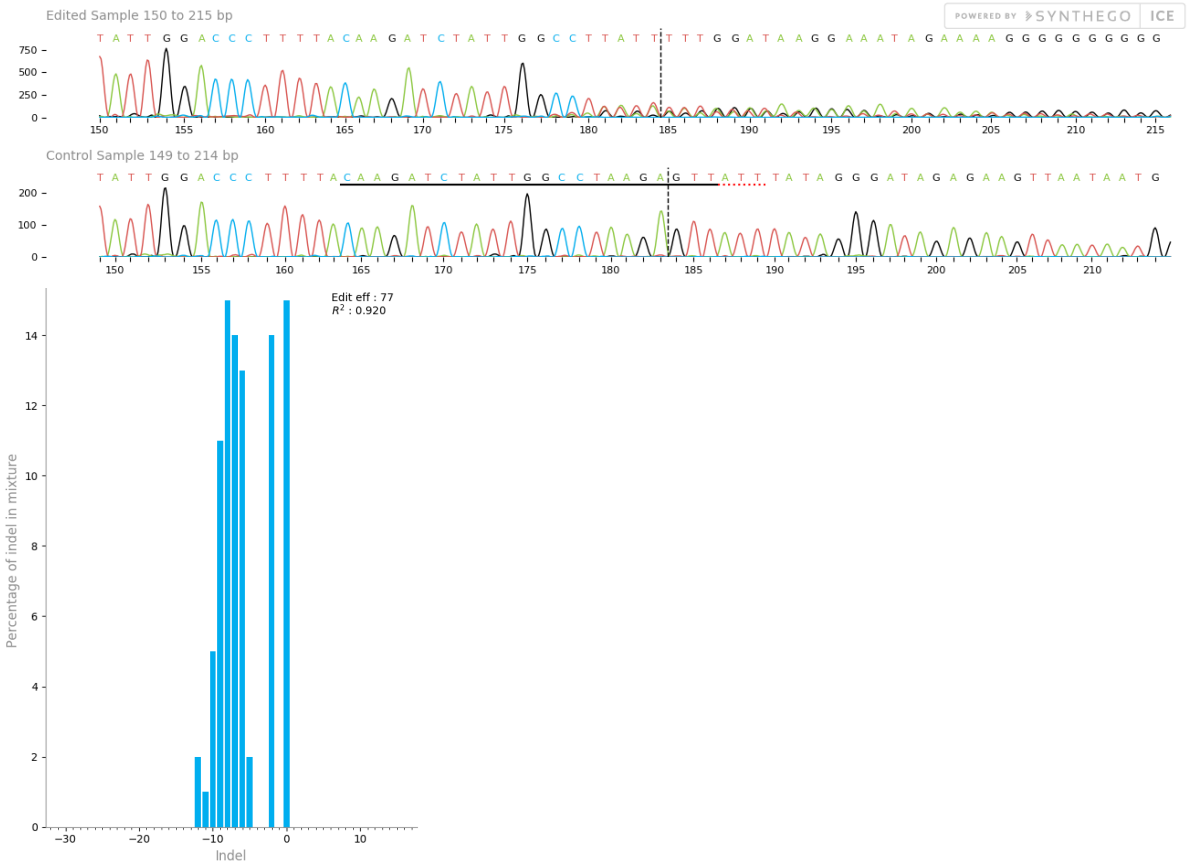
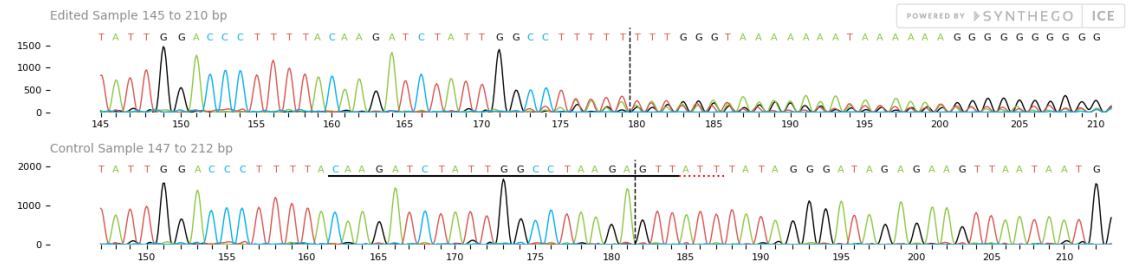


Figure S4. ICE analysis results of target site mutations in 35S::NIb *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT. Examples of sequencing chromatograms (top) and mutagenesis profiles (bottom) are shown.

MU237:



MU371:



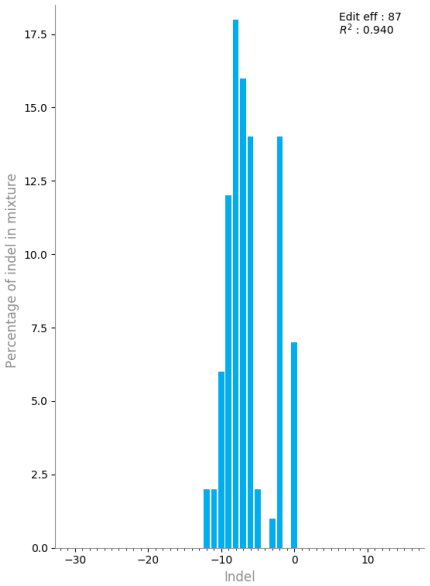


Figure S5. Representative leaves from wild-type *N. benthamiana* plants at 14 dpi inoculated with TEVΔNIb::LbCas12a alone (left), PVX::NIb:crFT alone (middle), or coinoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT (right).

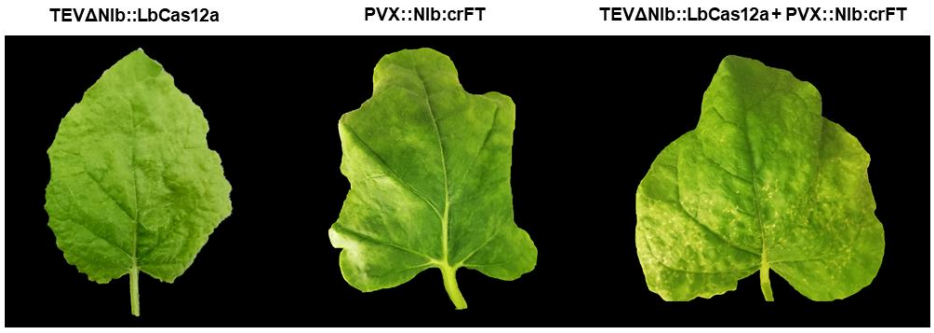


Table S1. *N. benthamiana* genes targeted by the CRISPR-Cas12a system. Note that PAM sequence recognised by LbCas12a nuclease is highlighted in grey.

Target gene	SolGenomics Accession No	Target site
NbFT	Niben101Scf01519g10008.1	**TTTA**CAAGATCTATTGGCCTAAGAGTT
NbXT1	Niben101Scf04205g03008.1	**TTTA**TATGTAGGTGTATTTGGAATTCT

Table S2. Primers used for the construction of recombinant viruses.

Construct element	Primer*	Primer sequence (5'→3')	Purpose
LbCas12a	D2521 (F)	attggtgtactcgcaaggggagaagATG AGCAAGCTGGAGAAG	Amplification of *LbCas12a* for TEVΔNIb::LbCas12a
	D2508 (R)	tctgaaaataaagattctcagtcgtGGCA TAGTCGGGGACATC	Amplification of *LbCas12a* for TEVΔNIb::LbCas12a
crFT	D3591 (F)	gaggtcagcaccagctagcaTAATTT CTACTAAGTGTAGATCAAG ATCTATTGGCCTAAG	Amplification of crFT for PVX::crFT
	D3592 (R)	gggaaacttaacaaaccctaATCTACA CTTAGTAGAAATTAAACTC TTAGGCCAATAGAT	Amplification of crFT for PVX::crFT

	D3712 (F)	ttttcagtaaTAATTTCTACTAAG TGTAGATCAAGATCTATTG GCCTAAG	Amplification of crFT for PVX::NIb:crFT
	D3713 (R)	gggaaacttaacaaaccctaATCTACA CTTAGTAGAAATTAAACTC TTAGGCCAATAGAT	Amplification of crFT for PVX::NIb:crFT
pBPVX:: crFT	D4036 (F)	TAATTTCTACTAAGTGTAG ATCAAGATCTATTGG	Amplification of pBPVX::crFT for PVX::crXT1:crTFL1:crFT
	D3536 (R)	TGCTAGCTGGTGCTGACC	Amplification of pBPVX::crFT for PVX::crXT1:crTFL1:crFT
crXT1	D4037 (F)	gaggtcagcaccagctagcaTAATTT CTACTAAGTGTAGATTATG TAGGTGTATTTGG	Amplification of crXT1 for PVX::crXT1:crFT
	D4038 (R)	ATCTACACTTAGTAGAAAT TAAGAATTCCAAATACACC	Amplification of crTFL1 3.1/14.1 for PVX::crXT1:crFT
NIb	D3710 (F)	gaggtcagcaccagctagcaTGGGGG AGAAGAGGAAATG	Amplification of TEV NIb cistron for PVX::NIb:crFT
	D3711 (R)	gtagaaattaTTACTGAAAATAA AGATTCTCAGTCG	Amplification of TEV NIb cistron for PVX::NIb:crFT

*F, forward primer; R, reverse primer

Table S3. Primer combinations used for the construction of recombinant viruses.

Construct	Primer names
TEVΔNIb::LbCas12a	D2521 + D2508 (template: pcDNA3.1::hLbCpf1)
PVX::crFT	D3591+ D3592 (no template)
PVX::crXT1:crFT	D4037 + D4038 D4036 + D3536 (template: pBPVX::crFT)
PVX::NIb:crFT	D3710 + D3711 (template: pGTEVa) D3712 + D3713

Table S4. Primers used for TEVΔNIb diagnosis by RT-PCR.

Primer*	Primer sequence (5'→3')	Purpose
D179 (R)	CTCGCACTACATAGGAGAATTAGAC	Reverse transcription of TEVΔNIb mRNA into cDNA
D178 (F)	AGTGGCACTGTGGGTGCTGGTGTTG	Analysis of TEVΔNIb infection
D211 (R)	GGCGCGGCGTCGACCTGGCGGACCCC TAATAG	Analysis of TEVΔNIb infection
D3604 (F)	ATAAGGAGACAGACTATCGGGC	Expression of *LbCas12a* nuclease in TEVΔN progeny
D3605 (R)	TGATCTGTCCGTGATTGTTCTC	Expression of *LbCas12a* nuclease in TEVΔN progeny

*F, forward primer; R, reverse primer, S, sequencing primer

Table S5. Primers used for Cas12a-crRNA gene editing analysis.

Gene	Primer name	Primer sequence (5'→3')	Purpose
NbFT	D3667 (F)	CTAGAAAACCTATGGCTATAAGGG	Amplification of a 550-nt DNA fragment flanking *NbFT* target region
	D3668 (R)	GTTCTCGAGAGGTATAATATAGGC	Amplification of a 550-nt DNA fragment flanking *NbFT* target region
	D3669	CACAAGCACGCATAGAAC	Sequencing of the 550-nt PCR product for the analysis of *NbFT* editing
NbXT1	JO16 JUN05 (F)	AACCACTTTTCCTCGTCGGAAA	Amplification of a 1286-nt DNA fragment flanking *NbXT1* target region
	JO16 JUN06 (R)	TAACTATTCAACTAAAGCTTCAAACAG	Amplification of a 1286-nt DNA fragment flanking *NbXT1* target region
	JO16 NOV05	TGTTTAATGAAGATTGTCTGG	Sequencing of the 1286-nt PCR product for the analysis of *NbXT1* editing

961 *F, forward primer; R, reverse primer
962
963